THE ROLE OF DRUG DISPOSITION GENES FOR VARIABILITY IN THE PHARMACOKINETICS OF ANTIRETROVIRAL DRUGS

Thesis submitted in accordance with the requirement of the University of Liverpool for the degree of Doctor of Philosophy

By

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This thesis is the result of my own work. The material contained within the thesis has not been presented, either wholly or in part, for any other degree or quantification.

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This research was carried out in the

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ABBREVIATIONS

µg	Microgram(s)
µl	Microlitre(s)
µM	Micromolar
ABC	ATP binding cassette
ACN	Acetonitrile
AIDS	Acquired immunodeficiency syndrome
AP	Apical
ART	Antiretroviral therapy
BL	basolateral
^{14}C	Carbon radioisotope
^{o}C	Degrees centigrade
C_{max}	Maximum plasma concentration
Caco-2	Human colon adenocarcinoma cell line
CAR	Constitutive androstane receptor
cART	Combination antiretroviral therapy
CCR5	CC chemokine receptor 5
CD4(+)	Helper T-lymphocyte antigen marker (expressing CD4)
cDNA	Complementary DNA
CNV	Copy number variation
CRF	Circulating recombinant forms
Ct	Cycle threshold
CXCR4	CXC chemokine receptor 4
CYP	Cytochrome P450
ddH_2O	Double-distilled water
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid

dsDNA	Double strand DNA
EDTA	Ethylamine-diaminetetra-acetic acid
EFV	Efavirenz
EI	Entry inhibitors
Env	Envelope glycoprotein
FBS	Foetal bovine serum
FDA	US Food and Drug Administration
g	gram(s)
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA
gp41	Glycoprotein 41
gp120	Glycoprotein 120
h	hour
^{3}H	Tritium
HAART	Highly active antiretroviral therapy
HBSS	Hanks Balance Salt Solution
HIV	human immunodeficiency virus
HPLC	High performance liquid chromatography
HQC	High quality control
kg	Kilogram(s)
L	Litre
LPV	Lopinavir
LQC	Low quality control
M	Molar
MDCK	Madin–Darby canine kidney
MeOH	methanol
mg	Milligram(s)
$MgCL_2$	Magnesium chloride
min	Minute(s)

ml	Millilitre
mM	Millimolar
MQC	Medium quality control
mRNA	Messenger RNA
ng	Nanogram(s)
nm	Nanometre(s)
nM	Nanomolar
nmol	Nanomole(s)
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
OATP	Organic anion-transporting polypeptide
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PI	Protease inhibitor
P-gp	P-glycoprotein
PK	pharmacokinetic
PXR	pregnane X receptor
qPCR	Quantitative Polymerase Chain Reaction
RFU	relative fluorescence units
RNA	Ribonucleic acid
RT	Reverse transcriptase
s	Second(s)
SD	Standard deviation
SNP	Single nucleotide polymorphism
TEER	Trans Epithelial Electrical Resistance
TDM	Therapeutic drug monitoring
UGT	Uridine 5’diphosphate-glucuronosyltransferase
v/v	Volume to volume ratio
VL	Viral load

ABSTRACT

The failure of highly active antiretroviral therapy may be due to pharmacological factors such as drug transporters and metabolism enzymes. Drug transporters and metabolism enzymes played complementary roles in drug absorption, distribution, metabolism and excretion by biotransformation and counter-transport, particularly in the intestine while nuclear receptors as transcription factors regulate the expression of drug transporters and metabolism enzymes. In this thesis, a positive correlation between nuclear receptors expression and the expression of ABC transporters and OATP transporters in intestine were observed while a negative correlation was found between the gene expression of nuclear receptors and cytochrome P450 enzymes in intestine. Single nucleotide polymorphisms in genes could potentially impact on gene expression of drug transporters and metabolism enzymes. The polymorphisms of nuclear receptors were associated with the expression of ABC transporters.

Drug concentrations have a high inter-individual variability in patients receiving the same dose of antiretroviral drugs, which could affect outcome of antiretroviral therapy. There are many factors that may affect plasma concentrations such as age, gender, body weight, ethnicity, genetic factors and so on. In a Ghanaian cohort, a negative correlation was found between the body weight and the EFV plasma concentration. Genetic factors such as the polymorphisms of cytochrome P450 enzymes also influenced efavirenz plasma concentrations. Meanwhile, efavirenz plasma concentrations were associated with the viral load in plasma within a UK cohort.

Nanomedicine involves new and promising technologies that may enable and improve the targeted delivery of antiretroviral drugs. The permeability of lopinavir in the Caco-2 cell line was improved by formulation of nanodispersions. However, the permeability of efavirenz was decreased for all nanodispersions in MDCKII and MDCKII-ABCB5 cell lines. Comparing efficiency of efavirenz nanodispersions transcellular permeability in MDCKII and MDCKII-ABCB5 cell lines indicated that ABCB5 is able to transport efavirenz when incubated as dissolved molecule or nanodispersion. It is support by the copy number variation of ABCB5 had no relationship with EFV plasma concentrations.

In summary, this thesis has attempted to determine the pharmacological factors influencing pharmacokinetics of HIV drugs, including drug transporters, metabolism enzymes and nuclear receptors. Data illustrating the factors that influence efavirenz plasma concentrations which are important for viral suppression were also generated. Furthermore, the nanodispersion technology is worthy of further study in order to improve drug delivery and drug distribution of antiretroviral drugs.

CHAPTER 1

General Introduction

Contents

1.1 Acquired Immunodeficiency Syndrome (AIDS)

Acquired immunodeficiency syndrome (AIDS) is a disease of the human immune system caused by the human immunodeficiency virus (HIV) (Sepkowitz, 2001). HIV destroys the immune system causing individuals to become susceptible to opportunistic infections and tumours. The transmission of HIV involves sexual intercourse (including oral and anal sex), contaminated blood transfusion and hypodermic needles, exchange between mother and baby during pregnancy, childbirth, breastfeeding or other exposure to bodily fluids (Albrektsson *et al.*, 2009; Mole *et al.*, 2011; Stock, 2011).

AIDS was first reported in 1981 by the U.S. Centres for Disease Control and Prevention (Gallo, 2006). Genetic research indicates that HIV originated in west-central Africa during the late nineteenth or early twentieth century (Sharp *et al.*, 2011). AIDS is now a pandemic disease in all over the world (Sharp *et al.*, 2011). The Joint United Nations Programme on HIV and AIDS (UNAIDS) estimated that there were 34 million people worldwide living with HIV/AIDS in 2010 from the report of UNAIDS 2011. There is a 27% increase compared with 26.2 million in 1999 from the global report of UNAIDS 2010 (UNAIDS, 2010). Meanwhile, 2.6 million people were newly infected with HIV in 2009 which is more than one fifth (21%) fewer than the estimated 3.2 million in 1997 (Table 1.1). The number of annual AIDS-related deaths worldwide is steadily decreasing from 2.1 million in 1997 to an estimated 1.8

million in 2009 (Table 1.1). Data have shown that the treatments for HIV decrease the number of people that die due to AIDS. Antiretroviral treatment reduces both the mortality and the morbidity of HIV infection, but there is no drug which can cure the patients and a vaccine has not been found (May *et al.*, 2011). Cost of therapy is another difficulty for treating HIV infection in many countries. Therefore the prevention of infection is a core objective to control the AIDS pandemic. Many programmes which attempt to slow the spread of the virus such as safe sex and needle-exchange have been promoted by the health organizations (Wasnik, 2011).

Table 1.1 the number of people infected with HIV and death due to AIDS

Year	**1970-2009**	**1990**	**1997**	**2009**	**Reference**
HIV infections	65,000,000	2,100,000	3,200,000	2,600,000	(UNAIDS, 2011)
AIDS-related Death	29,000,000	300,000	2,100,000	1,800,000	(UNAIDS, 2011)

1.2 Human Immunodeficiency Virus (HIV)

HIV is a lentivirus which is a member of the retrovirus family (Gonda, 1988; Volsky, 1990). Two types of HIV have been characterized: HIV-1 and HIV-2. HIV-1 is the virus that was initially discovered which is more virulent and infective (Gilbert *et al.*, 2003). HIV-1 infections are of global prevalence, while HIV-2 has a lower virulence and infectivity compared to HIV-1. Because of its relatively poor capacity for transmission, HIV-2 is largely confined to West Africa (Almaghrabi *et al.*, 2011).

1.2.1 Structure of HIV

HIV is larger than other viruses and its structure is different from other retroviruses. It is roughly spherical with a diameter of about 120 nm (Campbell *et al.*, 2001). Two copies of positive single-stranded RNA that codes for the virus's nine genes are enclosed by a conical capsid which is made by the viral protein p24 (Berthet-Colominas *et al.*, 1999). There are also some enzymes including reverse transcriptase, protease, ribonuclease and integrase in the capsid. A matrix composed of the viral protein p17 surrounds the capsid ensuring the integrity of the virion particle (Ellenrieder *et al.*, 2004). The virion is surrounded by a viral envelope which is a membrane with a phospholipid bilayer that comes from the host cell. An envelope glycoprotein (Env) composed of a cap made by three receptor binding domain gp120 and surface fusion protein subunit gp41 is embedded in the virus, which catalyzes virus entry and is a major target for therapeutic intervention and for neutralizing

antibodies (Buzon *et al.*, 2010)(Figure 1.1).

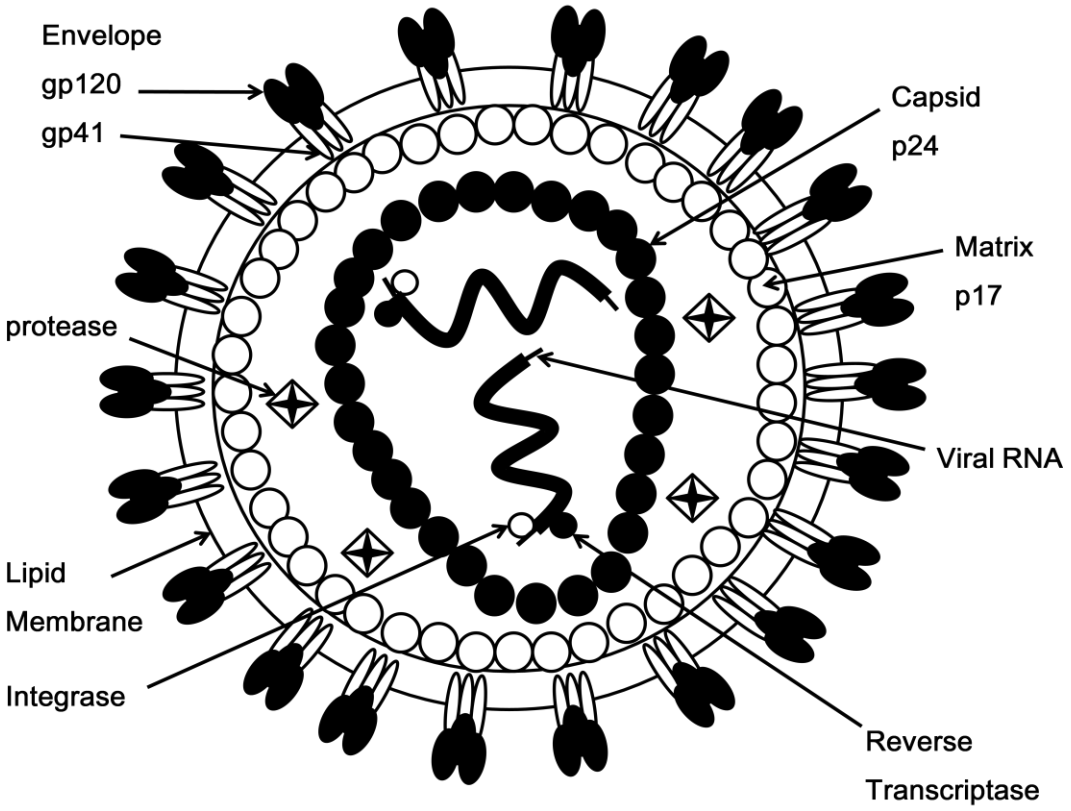


Figure 1.1 structure of HIV

1.2.2 HIV replication cycle

The replication cycle of HIV is shown in Figure 1.2. HIV can infect a variety of immune cells such as CD4+ T cells, macrophages, and the other cells with CD4 on its surface (Cunningham *et al.*, 2010).

When HIV gains entry to macrophages and CD4+ T cells, it is mediated through interaction of the virion envelope gp120 not only with the CD4 molecule but also with chemokine co-receptors CCR5 or CXCR4 on the target cells. The envelope complex of the virus undergoes a structural change after gp120 is bound with the CD4 protein. The chemokine binding domains of gp120 are then exposed and interact with CCR5 or CXCR4 on the host cell membrane (Fanales-Belasio *et al.*, 2010). When the N-terminal fusion peptide gp41 penetrates into the host cell membrane there is a change in the structure to a hairpin (Fanales-Belasio *et al.*, 2010). This loop structure brings the virus and cell membranes close together, allowing fusion of the membranes and subsequent entry of the viral capsid (Fanales-Belasio *et al.*, 2010).

After HIV fusion with the target cell, the virus RNA and various enzymes (including reverse transcriptase, integrase, ribonuclease, and protease) are injected into the host cell. Before transporting to the nucleus, the viral single-strand RNA is transcribed into double-stranded DNA by reverse transcriptase. The viral DNA is then integrated into the host cell genome by the enzyme integrase. This integrated DNA provirus may then lie dormant, in the latent stage of HIV infection (Zheng *et al.*, 2005).

Activation of the host cell results in the transcription of viral DNA into viral messenger RNA (mRNA). These viral mRNA are exported from the nucleus into the cytoplasm where they are translated into the viral proteins (Klimas *et al.*, 2008). These viral proteins are cleaved by HIV protease and localise with the viral RNA exported from the nucleus (Klimas *et al.*, 2008). The structural proteins are produced from the full-length mRNA which includes the virus genome. The genome then assembles with the proteins and is packaged into new virus particles, which bud through the host cell membrane. After acquiring a new envelope the virions are released into the extracellular space where they continue to mature (Fanales-Belasio *et al.*, 2010).

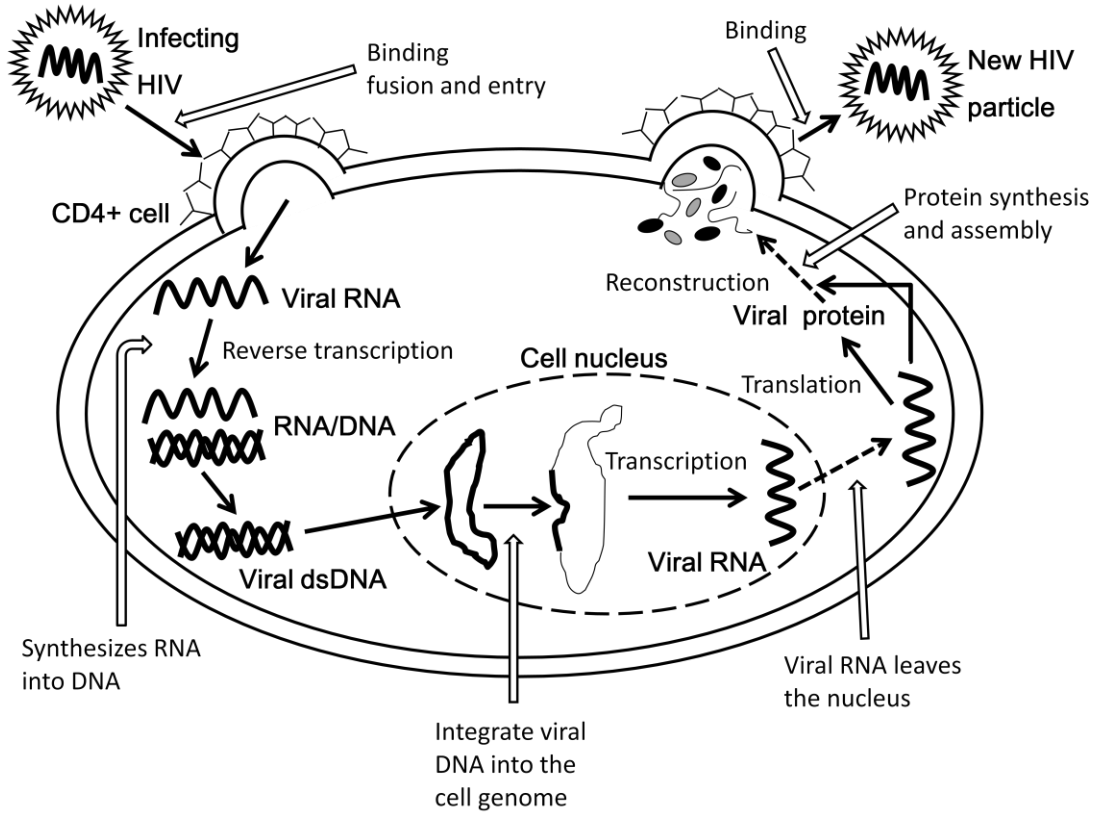


Figure1.2 HIV replication cycle dsDNA: double strand DNA

1.2.3 Variability of HIV

HIV is different from many viruses because it has very high variability. This diversity is a result of the "error-prone" mechanism of action of the virus enzyme reverse transcriptase (Sarafianos *et al.*, 2009), the very rapid viral replication and the occurrence of recombination processes between two or more different HIV viruses within the same infected individual (Fanales-Belasio *et al.*, 2010).

HIV can be divided into two major types, HIV-1 and HIV-2. HIV-1 is related to viruses found in chimpanzees and gorillas living in western Africa, while HIV-2 viruses are related to viruses found in sooty mangabeys. HIV-1 viruses may be further divided into groups, M (Major), O (Outlier) and N (non-M/non-O). The HIV-1 group M viruses predominate and are responsible for the AIDS pandemic. It is further subdivided into subtypes. Different subtypes can also be recombined as circulating recombinant forms (CRFs) such as CRF12_BF which is a recombination between subtypes B and F. Group O and Group N are rare but many HIV-positive samples have been found. It could not be detected by early versions of the HIV-1 test kits but more advanced HIV tests have now been developed to detect both Group O and Group N viruses (Aghokeng *et al.*, 2009). Likewise, HIV-2 can also be subdivided into a number of major groups. As of 2010, there are 8 known HIV-2 groups (A to H). Of these, only groups A and B are epidemic. Group A spread mainly in West Africa, but also to Angola, Mozambique, Brazil, India, and very limitedly to Europe and the US. Group B is mainly confined to West Africa (Hodges-Mameletzis *et al.*, 2011).

1.3 Antiretroviral drugs

1.3.1 Classes of drugs

Antiretroviral (ARV) drugs are primarily medications used for the treatment of HIV infection. They are broadly classified into five categories according to the phase of the HIV life-cycle (Table 1.2).

Entry inhibitors (EI)

Entry inhibitors interfere with the binding, fusion and entry of an HIV virion into a human cell. Enfuvirtide is the first and only HIV fusion inhibitor which is used in combination therapy for the treatment of HIV-1 infection (Cervia *et al.*, 2003). However, use of enfuvirtide is limited due to its availability only as a parenteral administration (Avelino-Silva *et al.*, 2012). CCR5 receptor antagonists are the first antiretroviral drugs which do not target the virus directly. Instead, they bind to the CCR5 receptor on the surface of the T-Cell and block viral attachment to the cell. Most strains of HIV attach to T-Cells using the CCR5 receptor. Therefore, the virus cannot gain entry to replicate when CCR5 is blocked by the antagonist. Currently the only available co-receptor antagonist is maraviroc, but others are in development. A limitation of this class of drug is that a HIV tropism test such as a Trofile Assay is required to ensure the patient doesn't harbour CXCR4-utilising strains of virus. This adds an additional cost to therapy

Reverse transcriptase inhibitors (RTIs)

RTIs are a class of antiretroviral drug used to treat HIV infection, which inhibit activity of reverse transcriptase, a viral DNA polymerase enzyme that retroviruses need to reproduce. There are two types of RTIs: Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs) and Non-nucleoside reverse transcriptase inhibitors (NNRTIs). NRTIs inhibit reverse transcription by being incorporated into the newly synthesized viral DNA strand as a "faulty" nucleotide. This causes a chemical reaction resulting in DNA chain termination. NNRTIs inhibit reverse transcriptase directly by binding to the enzyme and interfering with its function. Therefore, NRTIs are competitive inhibitors whereas NNRTIs are non-competitive inhibitors. NRTIs compose the first class of antiretroviral drugs developed, for example, zidovudine, didanosine, abacavir, tenofovir, apricitabine and so on (Table 1.2). Non-nucleoside reverse-transcriptase inhibitors (NNRTIs) are the third class of antiretroviral drugs that were developed and include efavirenz, nevirapine, delavirdine, etravirine and rilpivirine.

Protease inhibitors (PIs)

Protease inhibitors (PIs) are a class of medications used to treat or prevent infection by viruses, including HIV and Hepatitis C. PIs prevent viral replication by inhibiting the activity of HIV-1 protease, an enzyme used by the viruses to cleave nascent proteins for final assembly of new virions. In antiretroviral therapy, PIs include saquinavir, lopinavir, darunavir, atazanavir, ritonavir and so on (Table 1.2).

Antiretroviral PIs are substrates for multiple transport and metabolism proteins such as CYP3A isoforms (Josephson *et al.*, 2007), ABCB1, ABCC1 and ABCC2 (Jain *et al.*, 2007).

Integrase inhibitors

Integrase inhibitors are a class of antiretroviral drug designed to block the action of integrase, a viral enzyme that inserts the viral genome into the DNA of the host cell. The first integrase inhibitor approved by the U.S. Food and Drug Administration (FDA) was raltegravir, approved on October, 2007 (Steigbigel *et al.*, 2008). Raltegravir has an unusual pharmacokinetic - pharmacodynamic relationship and erratic absorption due to different solubility at different pH (Cattaneo *et al.*, 2012). Many other integrase inhibitors are now in development.

Maturation inhibitors

Maturation inhibitors inhibit the last step in gag processing in which the viral capsid polyprotein is cleaved, thereby blocking the conversion of the polyprotein into the mature capsid protein (p24). To date, these are experimental agents and no clinically used drugs in this class have been licensed (Martin *et al.*, 2008; Myriad-Genetics, 2010; Myriad-Genetics, 2008).

Entry inhibitors	Reverse transcriptase inhibitors		Protease inhibitors	Integrase inhibitors	Maturation inhibitors
	NRTIs	NNRTIs			
Enfuvirtide (INN)	Abacavir (ABC)	Efavirenz (EFV)	Saquinavir (SQV)	Raltegravir (RAL)	Bevirimat
Maraviroc (MVC)	Apricitabine	Nevirapine (NVP)	Ritonavir (RTV)	Elvitegravir *	Vivecon *
	Didanosine (ddI)	Delavirdine (DLV) **	Indinavir (IDV)	MK-2048 *	
	Emtricitabine (FTC)	Etravirine (ETV)	Nelfinavir (NFV)		
	Entecavir (ETV) **	Rilpivirine (TMC278)	Amprenavir (APV)		
	Lamivudine (3TC)		Lopinavir (LPV)		
	Stavudine (d4T)		Atazanavir (ATV)		
	Tenofovir (TDF)		Fosamprenavir (FPV)		
	Zalcitabine (ddC)		Tipranavir (TPV)		
	Zidovudine (ZDV)		Darunavir (DRV)		
	Adefovir *				

Table 1.2 Classifications of Antiretroviral Drugs (*: The drug has been not approved by the FDA for treatment of HIV; **: The drug is currently rarely used as an HIV drug)

1.3.2 Combination therapy

HIV treatment combines several drugs, typically three or four. This combination therapy is known as Highly Active Antiretroviral Therapy (HAART). The life cycle of HIV can divide into four stages: entry to the cell, replication and transcription, assembly, and release of additional viruses. The different classes of antiretroviral drugs act on different stages of the HIV life-cycle. Drug combinations can improve the effectiveness of the antiretroviral drugs and prevent multi-drug resistance emerging. The key aim of the antiretroviral combination therapy is to defend against resistance by suppressing HIV replication as much as possible.

Combinations of antiretroviral drugs can decrease resistance because if a mutation conferring resistance to one drug emerges, the other drugs can continue to suppress reproduction of that mutant. Combinations usually comprise two NRTIs and one NNRTI or PI (Ghosh *et al.*, 2011). Because the combinations of antiretroviral drugs are subject to positive and negative synergies, the total number of combination drugs must be limited. For example, the combination of didanosine and zidovudine was highly cytotoxic and mutagenic (Walker *et al.*, 2009).

1.4 Drug Disposition

The pharmacological action of oral drugs is dependent on adequate intestinal absorption, distribution to sites of action, and elimination by metabolic and excretory pathways. Drug metabolism reactions are generally divided into 2 phases, Phase I and Phase II. Phase I reactions are primarily mediated by the cytochrome P450 enzymes (Zollner *et al.,* 2010). The drugs are oxidized, reduced and hydrolysed by different enzymes. In Phase II reactions, the drugs (or Phase I metabolites) conjugate with some endogenous compounds, such as glucuronic acid, glutathione or sulphate so that they can be more readily excreted (Mottino *et al.,* 2000). Through targeted expression in organs (intestine, kidney, and liver), transporters also have a significant impact on the drug disposition process. Therefore, transporters, enzymes, the drug pKa, molecular weights, lipophilicity and other physicochemical properties can all affect the extent of drug absorption and access to target tissues.

1.4.1 Phase I enzymes

Cytochrome P450 is a very large and diverse superfamily of haem containing proteins found in all domains of life (Danielson, 2002). These enzymes are the major enzymes involved in drug metabolism and bioactivation, accounting for about 75% of the total metabolism (Wang *et al.*, 2010). CYP2B6 can metabolise approximately 8% of clinically used drugs, including efavirenz, nevirapine, cyclophosphamide, ifosfamide, tamoxifen, ketamine, artemisinin, bupropion, sibutramine, and propofol (Mo *et al.*, 2009). CYP3A4 metabolises approximately 50% of the marketed drugs including antiretroviral agents such as the PIs and certain NNRTIs (Kumar *et al.*, 2011). Many substances are bioactivated by Cytochrome P450 enzymes to form their active compounds. In HAART, the NNRTI efavirenz is a substrate of CYP2B6 and nevirapine is a substrate for both CYP2B6 and CYP3A4. The second-generation NNRTI etravirine is also a substrate of CYP3A4 (Tseng *et al.*, 2010).

Several PIs such as indinavir, darunavir, lopinavir, saquinavir, atazanavir, tipranavir and ritonavir are all predominantly metabolised by CYP3A4 (Kumar *et al.*, 2011). Cytochrome P450 enzymes are present in many tissues of the body including hepatocytes, lymphocytes and the mucosa of the gastrointestinal tract (Fagiolino *et al.*, 2011). CYP3A4 constitutes 80% of total intestinal CYP protein expression, followed by CYP2C at 18%, and that CYP2B6 expression is relatively minor – however this will still be important if the absorption and distribution of a drug is heavily dependent on CYP2B6 (Thelen *et al.*, 2009).

1.4.2 Influx and Efflux Transporters

Both influx and efflux transporters are important in determining oral drug disposition by controlling absorption and bioavailability. See Figure 1.3 and 1.4 for their localization in enterocytes and hepatocytes and Table 1.3 for examples of substrates for those transporters expressed in tissue. The major influx transporters responsible for xenobiotic transport belong to the two solute carrier superfamilies. In this thesis, we focused on the Organic anion-transporting polypeptide (OATP) superfamily transports. OATP transporters are plasma membrane transport proteins that mediate the active cellular influx of a variety of amphipathic compounds (Pacyniak *et al.*, 2010). Substrates include bile salts, steroid conjugates, thyroid hormones, anionic oligopeptides and several therapeutic drugs and other xenobiotics (Leuthold *et al.*, 2009). PIs but not NNRTIs have also been shown to be substrates for these transporters (Hartkoorn *et al.*, 2010). OATP transporters are expressed in various cells and tissues such as the liver, kidneys, intestine and the blood–brain barrier (Table 1.3), and therefore, may significantly affect the pharmacokinetics and distribution of their substrate drugs.

Efflux transporters expressed in the intestine and liver include P-glycoprotein (P-gp, ABCB1), bile salt export pump (BSEP, ABCB11), multidrug resistance proteins (MRP1- 6, ABCC1-6), and breast cancer resistance protein (BCRP, ABCG2), all members of the ATP-Binding Cassette superfamily (Choudhuri *et al.*, 2006; Kosters *et al.*, 2008). Members of this superfamily use ATP as an energy source, allowing them

to pump substrates against a concentration gradient. These proteins transport various compounds (e.g. lipids and sterols, ions and small molecules, drugs, and large polypeptides) across extra- and intracellular membranes (Dean *et al.*, 2001). Therefore, the ABC transporters play an important role in pharmacokinetics of numerous drugs through their expression in the intestine, liver, and kidney (Table 1.3). They do this by limiting absorption in the gut and so reducing the bioavailability, controlling the drug elimination into bile, urine, and faeces, and restricting the drug distribution to modulate the effectiveness of the drug therapy (Albermann *et al.*, 2005). They are also known to play a crucial role in conferring the development of multidrug resistance in cancer (Calcagno *et al.*, 2008). Transporters can also be involved in drug-drug interactions, which can potentially increase or decrease drug concentrations in plasma and/or tissues (Calcagno *et al.*, 2007; Ding *et al.*, 2004; Staud *et al.*, 2010). Activity of ABC-transporters is highly variable and may be altered within hours by inhibitors and within days by inducing agents. Also, genetic influences or endogenous factors like sex-hormones (Calcagno *et al.*, 2008) or cholesterol (Aarnoudse *et al.*, 2008) might influence transporter activity. Many ABC transporters are expressed in intestine, for example ABCG2 was identified as the most abundantly expressed transporter in the intestinal mucosa and ABCB1, ABCC1 and ABCC2 were highly expressed in jejunal tissue (Hilgendorf *et al.*, 2007). ABCC10 are expressed in a wide range of normal tissues including colon (Oguri *et al.*, 2008). A number of efflux transporters belonging to the ABC superfamily, are able to transport antiretroviral drugs. ABCG2 has significant inhibition for antiretroviral drugs such as lopinavir, nelfinavir, delavirdine,

efavirenz, saquinavir and so on (Weiss *et al.*, 2007a). ABCB1 have been implicated in the efflux of some antiretroviral drugs (Chandler *et al.*, 2007). ABCC1, ABCC2, and ABCC3 inhibit antiretroviral drugs including Nucleoside, Nucleotide, and Non-Nucleoside Reverse Transcriptase Inhibitors (Weiss *et al.*, 2007b). ABCC10 is substrate for antiretroviral drugs (e.g. zalcitabine, tenofovir and nevirapine) (Liptrott *et al.*, 2012). These proteins may thus influence antiretroviral drug therapy by their effect on pharmacokinetics and by promoting drug resistance (Borst *et al.*, 2004). In my thesis, we focused on transporters for which NNRTIs and PIs were substrates.

1.4.3 Nuclear receptors

Nuclear receptors are a class of proteins found within the interior of cells that are responsible for sensing the presence of steroid and thyroid hormones and certain other molecules. In response, these receptors work in concert with other transcription factors to regulate the expression of specific genes, thereby controlling development, homeostasis, and metabolism within the organism. Nuclear receptors have the ability to directly bind to DNA and regulate the expression of adjacent genes, hence these receptors are classified as transcription factors (Olefsky, 2001).

The pregnane X receptor (PXR) is a nuclear receptor whose primary function is to sense the presence of foreign toxic substances and, in response, up-regulate the expression of proteins (e.g. enzymes and transporters) involved in the detoxification and clearance of these substances from the body (Kliewer *et al.*, 2002). The constitutive androstane receptor (CAR) is also a member of the nuclear receptor superfamily, which also regulates the transcription of target genes involved in drug metabolism such as the cytochrome P450 family and transporters (Kakizaki *et al.*, 2008).

PXR and CAR bind as heterodimeric complexes with the retinoid X receptor to response elements in the regulatory regions of the induced genes. PXR is directly activated by xenobiotic ligands, whereas CAR is involved in a more complex and less well understood mechanism of signal transduction triggered by drugs. One of the

primary targets of PXR activation is the induction of CYP3A4, an important phase I oxidative enzyme (Sandanaraj *et al.*, 2008). In addition, PXR up regulates the expression of phase II conjugating enzymes such as glutathione S-transferase (Swales *et al.*, 2011) and phase III transport uptake and efflux proteins such as ABCB1, ABCC2, ABCC3 and OATP2 (Kast *et al.*, 2002; Staudinger *et al.*, 2001; Teng *et al.*, 2003). PXR can up-regulate both ABC transporters (ABCB1 and ABCC2) and OATP transporters by rifampicin (Peters *et al.*, 2011). Meanwhile, PXR also directly influences the basal expression levels of the ABC transporters (ABCB1, ABCC1, ABCC2 and ABCC10) and OATP transporters in liver and intestine (Albermann *et al.*, 2005; Svoboda *et al.*, 2011; Xu C., 2005). CAR appears to be responsible for induction of ABCC2 mRNA in human hepatocytes (Kast *et al.*, 2002). CAR is co-expressed with and co-regulates ABC transporters (ABCB1, ABCC1, ABCC2 and ABCC10) and OATP transporters in intestine and liver (Svoboda *et al.*, 2011; Xu C., 2005). CAR are also known to regulate gene expression of CYP2B6 and CYP3A4 (Hasegawa *et al.*, 2010; Rhodes *et al.*, 2011) and its expression correlates with CYP2B6 gene expression in liver (Chang *et al.*, 2003).

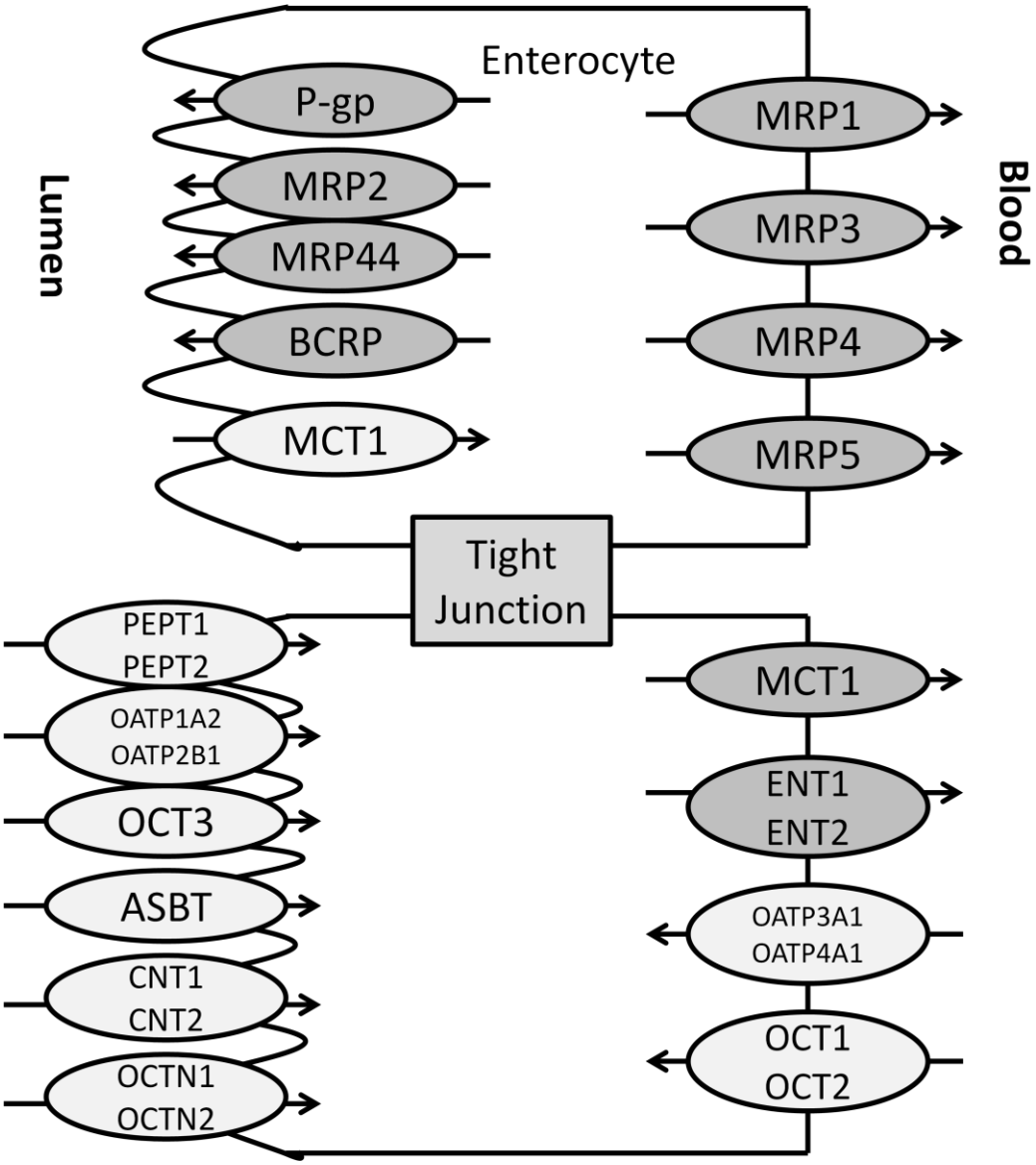


Figure 1.3 Enterocyte with intestinal influx (white) and efflux (black) transporters. P-glycoprotein (P-gp), Multidrug resistance protein (MDR), Multidrug resistance associated protein (MRP), Breast cancer resistance protein (BCRP), Monocarboxylate transporter protein (MCT), Peptide transport protein (PEPT), Organic anion transporting polypeptide (OATP), Organic cation transporter (OCT), apical sodium-dependent bile acid transporter (ASBT), Concentrative nucleotide transporter (CNT), Electroneutral organic cation transporter (OCTN), Equilibrative nucleoside transporter (ENT). Adapted from Custodio *et al* (Custodio *et al.*, 2008).

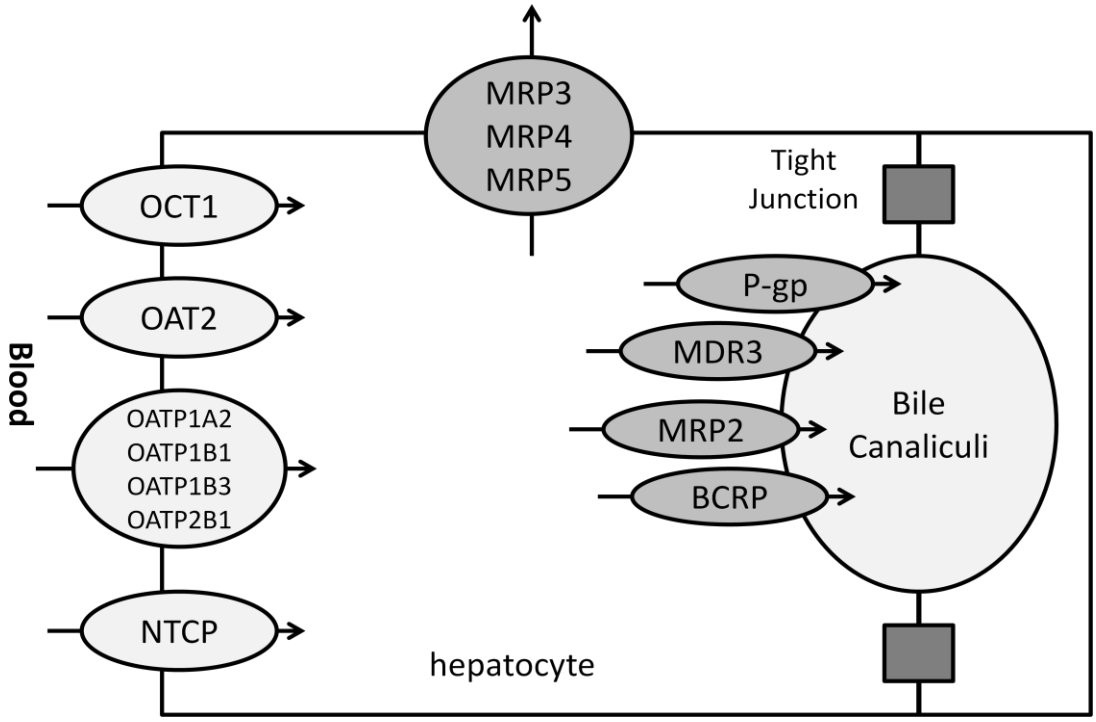


Figure 1.4 Hepatocyte with hepatic influx (white) and efflux (black) transporters. P-glycoprotein (P-gp), Multidrug resistance protein (MDR), Multidrug resistance associated protein (MRP), Breast cancer resistance protein (BCRP), Bile salt export pump (BSEP), Organic cation transporter (OCT), Organic anion transporter (OAT), Organic anion transporting polypeptide (OATP), sodium-taurocholate cotransporting polypeptide (NTCP). Adapted from Custodio *et al (Custodio et al., 2008)*

Genes	Tissues	Antiretroviral drug substrates	references
CYP2B6	liver, brain	efavirenz, nevirapine	(Lee et al., 2006; Mo et al., 2009)
CYP3A4	liver, intestine, brain	protease inhibitors, maraviroc, efavirenz, nevirapine, etravirine	(Eagling et al., 1999; Ghosh et al., 2011a; Hu et al., 2005; Le et al., 2012; Sevrioukova et al., 2010; Tseng
ABCB1	liver, intestine, kidney, brain	protease inhibitors, raltegravir, vicriviroc elvitegravir, maraviroc	(Giraud et al., 2010; Ho et al., 2005; Walker et al., 2008; Zembruski et al., 2011)
ABCC1	ubiquotious	protease inhibitors	(Giraud et al., 2010; Ho et al., 2005)
ABCC2	liver, intestine, kidney	protease inhibitors	(Giraud et al., 2010; Ho et al., 2005)
ABCC10	liver, intestine	nevirapine, tenofovir	(Liptrott et al., 2012; Pushpakom et al., 2011)
OATP1A2	brain, kidney, liver, intestine, testis	protease inhibitors	(Gao et al., 2000; Glaeser et al., 2007; Hartkoorn et al., 2010; Kullak-Ublick et al., 1995; Lee et al., 2005)
OATP1B1	liver	protease inhibitors	(Hartkoorn et al., 2010; Ho et al., 2005)
OATP1B3	liver	protease inhibitors	(Hartkoorn et al., 2010; Ho et al., 2005)
OATP1C1	brain, testis, kidney	unknown	(Liedauer et al., 2009; Pizzagalli et al., 2002)
OATP2A1	organ of human body	unknown	(Schuster, 1998)
OATP2B1	liver, placenta, intestine, brain	unknown	(Kobayashi et al., 2003; Kullak-Ublick et al., 2001; St-Pierre et al., 2002; Tamai et al., 2000)
OATP3A1	testis, heart, brain	unknown	(Adachi et al., 2003)
OATP4A1	placenta	unknown	(Sato et al., 2003)
OATP4C1	kidney	unknown	(Svoboda et al., 2011)
OATP5A1	breast cancer	unknown	(Liedauer et al., 2009)
OATP6A1	testis	unknown	

Table 1.3 Tissue distributions of disposition genes and their drug substrates

1.5 Pharmacokinetics

Pharmacokinetics is a fundamental scientific discipline that underpins applied therapeutics. It describes the movement of a drug around the body and shows what the body is doing to the drug. Understanding of pharmacokinetics should help to appreciate how dosage regimens are devised, design a dosage regimen to the individual requirements of a patient and determine what is wrong when a patient fails to respond to treatment. It is also important to find out why a drug has caused toxicity and elucidate the mechanisms of drug interactions. Pharmacokinetics provides a mathematical basis to assess the time course of drugs and their effects in the body. It enables the following processes to be quantified: Absorption, Distribution, Metabolism and Excretion (ADME). Bioavailability, clearance and volume of distribution are three key parameters for pharmacokinetics.

The variability in pharmacokinetics may arise from several factors such as genetics, disease (e.g. fever, congestive heart failure, gastrointestinal and hepatic), age, body weight, gender, food, environment (e.g. smoking and pollutants) and co-administered drugs (Becker *et al.*, 2002; Lane *et al.*, 2012; van den Anker *et al.*, 2011). After a drug is administered orally, the plasma concentrations change over a period of time. The marked inter-individual variability in drug concentrations and the relationship between plasma drug concentrations and efficacy or toxicity make antiretroviral drugs candidates for therapeutic drug monitoring (Back *et al.*, 2006). Intracellular concentrations of NNRTIs and PIs may be an important determinant of antiviral

activity, and the pharmacokinetics of intracellular drug accumulation is likely to impact upon efficacy and toxicity (Owen *et al.*, 2004). The plasma drug levels of PIs and NNRTIs correlate with viral suppression and drug toxicity (Fraaij *et al.*, 2005). Most of the PIs display poor pharmacokinetic properties because they are substrates of cytochrome P450 3A4 (Bazzoli *et al.*, 2010) and transporters (Bierman *et al.*, 2010; Hartkoorn *et al.*, 2010). Variability in the plasma concentration of efavirenz and nevirapine is associated with the therapeutic effect on patients (Darwich *et al.*, 2008). Plasma efavirenz levels are associated with efavirenz efficacy (Yimer *et al.*, 2011) and central nervous system toxicity (van Luin *et al.*, 2009).

1.6 Pharmacogenetics

Pharmacogenetics is the branch of pharmacology which deals with the influence of genetic variation on drug response in patients by correlating gene expression or single nucleotide polymorphisms (SNP) with a drug's pharmacokinetics efficacy or toxicity. By doing so, pharmacogenetics aims to develop rational means to optimize drug therapy, ensuring maximum efficacy with minimal adverse effects. Such approaches promise the advent of "personalized medicine", in which drugs and drug combinations are optimized for each individual's unique genetic makeup.

CYP2B6 is responsible for the metabolic clearance of efavirenz and CYP2B6 516TT are associated with efavirenz pharmacokinetics in the cohort of the German (Wyen *et al.*, 2011). CYP2B6 516TT genotype increased the plasma efavirenz and nevirapine concentrations in Thai cohort (Uttayamakul *et al.*, 2010). However, in other studies, CYP2B6 516GG genotype decreased the plasma efavirenz concentrations but not nevirapine, while CYP3A4 polymorphisms had no significant impact on plasma efavirenz or nevirapine concentrations in a Chinese patient population (Chen *et al.*, 2010). UGT2B7*1a and CYP2A6 (*9 and *17) carrier status increased efavirenz plasma concentrations (Kwara *et al.*, 2009). Finally, CAR-CC (rs2307424) also correlates with efavirenz plasma concentrations in German cohort (Wyen *et al.*, 2011).

Previous pharmacogenetics studies have not provided sufficient data to explain variability of PI pharmacokinetics in patients (Granfors *et al.*, 2006; van Erp *et al.*,

2007). Ritonavir is an inhibitor for many of the proteins that have been identified as pharmacogenetic targets and ritonavir is usually given in combination with other antiretroviral drugs. For example, CYP3A5 is an important determinant of the pharmacokinetics of saquinavir but CYP3A polymorphisms do not influence of PI pharmacokinetics. This may be because ritonavir is a potent inhibitor of CYP3A4/5 (Josephson *et al.*, 2007). PIs are also substrates for ABCB1 and ritonavir is also an inhibitor of ABCB1. Therefore, it is difficult to explain why ABCB1 polymorphisms would influence protease inhibitor exposure (Jain *et al.*, 2007). The ability of ritonavir to competitively inhibit transporters and metabolising enzymes has lead to its use as a "booster" during therapy with PIs. OATP transporters such as OATP1B1, OATP1B3 and OATP1A2 have been reported to use PIs as substrates and OATP1B1 SNPs are associated with lopinavir pharmacokinetics (Hartkoorn *et al.*, 2010; Su *et al.*, 2004). Genome wide association study show the PGx link between OATP1B1 expression and the risk of statin-induced myopathy for simvastatin (Link *et al.*, 2008). Currently there are no guidelines regarding fold change in AUC that is indicative of clinically significant genetic associations. However, for a drug-drug interaction, higher than 5-fold difference in AUC is defined by the FDA as a strong interaction.

A PXR-TT (PXR C63396T) increased Atazanavir clearance through an effect on hepatic PXR expression and therefore expression of its target genes (e.g. CYP3A4, ABCB1and OATP1B1), which are known to be involved in Atazanavir clearance (Schipani *et al.*, 2010). PXR*1B haplotype constitution could be important in

decreasing doxorubicin clearance through reduced hepatic mRNA expression of PXR and its downstream targets (Sandanaraj *et al.*, 2008). PXR and CAR polymorphisms regulate the expression of OATP1B (OATP1B1 and OATP1B3) transporters both *in vitro* and *in vivo* (Meyer zu Schwabedissen *et al.*, 2009).

As PXR regulates cytochrome P450 enzymes, the PXR polymorphisms have revealed not only changes in PXR expression and activity, but also effects on CYP3A4 gene expression (Svard *et al.*, 2010). PXR polymorphism was associated with significantly lower expression of CYP3A4 in liver tissues (Sandanaraj *et al.*, 2008). In antiretroviral therapy, several drugs including darunavir, fosamprenavir, lopinavir, nelfinavir, tipranavir, efavirenz, and abacavir increased the expression of cytochrome P450 enzymes through PXR (Svard *et al.*, 2010).

1.7 Aim of Thesis

It is well recognised that different patients respond in different ways to the same medication. These differences are often greater among members of a population than they are within the same person at different times (or between monozygotic twins). The existence of large population differences with small intra-patient variability is consistent with inheritance as a determinant of drug response. Although many factors influence drug response, including age, organ function, concomitant therapy, drug–drug interactions, and the nature of the disease, it is estimated that genetics can account for 20–95 percent of the variability in drug disposition and response (Kalow *et al.*, 1999). Once a drug is administered, it is absorbed and distributed to its site of action, where it interacts with targets (such as receptors and enzymes), undergoes metabolism, and is then excreted. Each of these processes influences the drug response and can potentially involve clinically significant genetic variation. The field of pharmacogenetics began with a focus on drug metabolising enzymes (Meyer, 2004; Weinshilboum, 2003), and then extended to membrane transporters that influence drug absorption, distribution, and excretion (Evans *et al.*, 2003; Fischer *et al.*, 2005; Gerloff, 2004; Kerb *et al.*, 2001; Kim, 2002; Roden *et al.*, 2002).

The main aims of this thesis are to investigate the role of drug disposition genes for variability in the pharmacokinetics of antiretroviral drugs. The design of the studies was as follows:

- Evaluation of the impact of nuclear receptor expression and single nucleotide polymorphisms on the expression of drug transporters in intestine
- Investigation of the impact of nuclear receptor expression and single nucleotide polymorphisms on the expression of cytochrome P450 enzymes in intestine
- Determination of the influence of drug concentration on the viral load in patients receiving highly active antiretroviral therapy
- Assessment of the effects of single nucleotide polymorphisms and copy number variations on efavirenz plasma concentrations
- Comparison of nanodispersions and aqueous solutions of antiretroviral drugs with respect to transcellular permeability in Caco-2, MDCKII and MDCKII-ABCB5 cell lines

CHAPTER 2

Impact of nuclear receptors expression and single nucleotide polymorphisms on the gene expression of drug transporters in intestine

Contents

2.1 INTRODUCTION

Since the discovery of P-gp in 1976 (Juliano *et al.*, 1976), the importance of drug transporters in influencing the pharmacokinetics of orally dosed drugs has become increasingly evident (Fromm, 2000; Landowski *et al.*, 2003; Mizuno *et al.*, 2002). Drug transporters in both the gut and the liver can help control access of drugs to systemic circulation by dictating the amount of drug that enters the body from the gut lumen and influencing how much drug escapes first pass metabolism in both gut and liver. Distribution of therapeutic compounds *in vivo* can be affected by transport across cell membranes by drug transporters. For example, ABC transporters can actively restrict drug distribution to the site of action by drug efflux from the cell via an ATP-dependant mechanism. Probably the best studied ABC transporter is ABCB1 (P-glycoprotein). ABCB1 is capable of expelling many hydrophobic compounds including chemotherapeutic agents (e.g. etoposide, doxorubicin, and vinblastine), immunosuppressive agents (e.g. cyclosporine A, methotrexate) and HIV-type 1 antiretroviral therapy agents like protease inhibitors and non-nucleoside reverse transcriptase inhibitors (NNRTI). ABCB1 is extensively distributed and expressed in many tissues of the body including in the kidney on the apical side of the intestinal lumen, the apical side of the bile canaliculus in liver hepatocytes, the apical membrane of proximal tubule epithelial cells and the apical side of the capillaries in the blood brain barrier (BBB). (Carl *et al.*, 2010; Chai *et al.*, 2011; Daood *et al.*, 2008; He *et al.*, 2011; Jung *et al.*, 2011; Wright *et al.*, 2004).

ABCC1 is expressed in the liver, kidney, intestine, brain and other tissues (He *et al.*, 2011) and it is localized to the basolateral membrane in intestinal epithelium (Beedholm-Ebsen *et al.*, 2010), hepatocytes (Fülöp *et al.*, 2008), Henle loop of kidney (Wright *et al.*, 2004) and choroid plexus epithelium of the blood-cerebrospinal fluid barrier (Daood *et al.*, 2008). ABCC1 is associated with tumour resistance which is often caused by an increased efflux and decreased intracellular accumulation of natural product anticancer drugs and other anticancer agents (He *et al.*, 2011). ABCC2 is expressed mainly in the apical membrane of liver canaliculi, renal proximal tubules, gut enterocytes, placenta, and brain-blood barrier (Lai *et al.*, 2007). ABCC10 is highly expressed in liver, intestine, and peripheral blood cells (Liptrott *et al.*, 2012) and localizes to the basolateral cell surface (Malofeeva *et al.*, 2012). Similar to ABCC1, ABCC2 and ABCC10 are also associated with the resistance of anticancer drugs (Chen *et al.*, 2009; Zhou *et al.*, 2009). In my thesis, we focused on transporters for which NNRTIs and PIs were substrates. Although ABCG2 was identified as the most abundantly expressed transporter in the intestinal mucosa (Hilgendorf *et al.*, 2007) ABCG2 has been shown to transport NRTIs but this wasn't a focus of my thesis (Weiss *et al.*, 2007).

OATP transporters are expressed in various cells and tissues, some members show a more restricted pattern (well-studied OATP1B1/OATP1B3 in liver, OATP4C1 in kidney, and OATP6A1 in testis) (Svoboda *et al.*, 2011). Intestinal epithelia contain in their apical (luminal) membrane several uptake transporters including one or more

members of the organic anion transporting polypeptide (OATP) family (Giacomini *et al.*, 2010). Three members of the OATP family (OATP1B1, OATP1B3 and OATP2B1) localize in the basolateral membrane of human hepatocyte, one member (OATP4C1) in kidney proximal tubule epithelia and two members (OATP1A2 and OATP2B1) in apical membrane of brain capillary endothelial cells (Giacomini *et al.*, 2010).

Nuclear receptors, including CAR and PXR, regulate hepatic and intestinal transporters in response to exogenous and endogenous activators (Martin *et al.*, 2008). PXR can modulate and/or up-regulate both ABC transporters (ABCB1 and ABCC2) and OATP transporters by rifampicin (Peters *et al.*, 2011). Meanwhile, PXR also directly influences the basal expression levels of the ABC transporters (ABCB1, ABCC1, ABCC2 and ABCC10) and OATP transporters in liver and intestine, in a cell- and tissue-specific way (Albermann *et al.*, 2005; Svoboda *et al.*, 2011; Xu C., 2005). CAR is co-expressed with and co-regulates ABC transporters (ABCB1, ABCC1, ABCC2 and ABCC10) and OATP transporters in intestine and liver, also in a cell- and tissue-specific way (Svoboda *et al.*, 2011; Xu C., 2005).

Polymorphisms within regulatory sequences at the 5’ and 3’ ends of the gene may affect transcription by altering the structure of transcription factor binding sites (Scotto, 2003). Polymorphisms occurring in introns may affect mRNA splicing, if at the ends of the intron, or the structure of enhancers or silencers (Tabara *et al.*, 2002).

A PXR polymorphism (*PXR63396 C>T*) influences unboosted atazanavir clearance through an effect on hepatic PXR expression and therefore expression of its target genes (e.g. CYP3A4, ABCB1and SLCO1B1), which are known to be involved in atazanavir clearance (Schipani *et al.*, 2010). PXR haplotype constitution could be important in influencing interindividual and interethnic variations in disposition of its putative drug substrates through reduced hepatic mRNA expression of PXR and its downstream targets (Sandanaraj *et al.*, 2008). PXR and CAR polymorphisms regulate the expression of OATP1B (OATP1B1 and OATP1B3) transporters both *in vitro* and *in vivo* (Meyer zu Schwabedissen *et al.*, 2009).

The aim of this chapter was to investigate the relationship between nuclear receptors (PXR and CAR) gene expression and the expression of ABC transporters (ABCB1, ABCC1, ABCC2 and ABCC10) and OATP transporters (OATP1A2, OATP1B1, OATP1B3, OATP1C1, OATP2A1, OATP2B1, OATP3A1, OATP4A1, OATP4C1, OATP5A1 and OATP6A1) in human intestine. The effect of nuclear receptor polymorphism (*PXR*44477 *T>C* [rs1523130], *PXR63396 C>T* [rs2472677], *PXR*69789 *A>G* [rs7643645] and *CAR540 C>T* [rs2307424]) on the gene expression of these ABC transporters and OATP transporters was also determined.

2.2 METHODS

2.2.1 Materials

TRIzol reagent, chloroform and nuclease free water were purchased from Sigma-Aldrich (Poole, UK). Isopropyl alcohol and ethanol were obtained from Fisher Scientific (Loughborough, UK). TaqMan reverse transcription (RT) kits were purchased from Applied Biosystems (Warrington, UK). Primers and probes for real-time PCR based gene expression assay and allelic discrimination assay were obtained from Assays by Design Service, Applied Biosystems (Warrington, UK). qPCR master mix was obtained from ABgene (Epsom, UK). Whole blood DNA extraction kit purchased from QIAGEN (West Sussex, UK).

2.2.2 Patients Data

84 patients were recruited from the Royal Liverpool University Hospital. Patient characteristics, including sex, age and ethnicity, were obtained from medical records. The group comprised 47 male and 36 female patients (one person no record). The average age was 60.3 years old with a range of 17 to 88 years old. The ethnicity of these patients included White British, African and others. The intestinal biopsy samples from duodenum were used to investigate the human gene expression and the matched blood samples were used for genotype. Insufficient biopsy material was available to quantify protein expression.

2.2.3 Extraction and preparation of mRNA samples

The D2 intestinal biopsy samples were homogenized in 1ml of Trizol reagent per 50-100mg of tissue by using a glass-Teflon homogenizer. The sample volume did not exceed 10% of the volume of Trizol reagent used for homogenization.

Homogenized tissue samples were incubated for 5 minutes at 37℃ to permit the complete dissociation of nucleoprotein complexes. 0.2ml of chloroform per ml of Tri reagent was added. Sample tubes were shaken vigorously for 15 seconds and incubated at 37℃ for 2 minutes. Samples were centrifuged at 12,000×g for 15 minutes at 4℃. The aqueous phase containing total RNA was transferred to a fresh tube. RNA was precipitated by mixing with 0.5ml of isopropyl alcohol per ml of Tri reagent used. The samples were incubated at 37℃ for 10 minutes and RNA harvested by centrifugation at 12,000×g for 10 minutes at 4℃. RNA was washed with 75% ethanol. RNA pellets were briefly air dried. Then dissolved in 10µl nuclease free water and stored at -80℃.

2.2.4 Reverse Transcription of mRNA samples

RNA sample concentration and purity were assessed by spectrophotometry (NanoDrop 100 Spectrophotometer, Thermo Scientific, Wilmington, USA). cDNA was generated form mRNA by reverse transcription. Reverse transcription reaction contained: 10×TaqMan RT buffer (5μl), 25mM $MgCl_2$ (11μl, final conc. 5.5mM), dNTP mix (10μl, 500μM each), Random Hexamers (2.5μl), RNase inhibitor (1μl, 0.4U/μl), Reverse Transcriptase (1.75μl, 1.25U/μl) and RNA (2μg). Reaction mixtures were made up to 50μl with RNase free water. Reverse transcription reaction occurred by using the Eppendorf Mastercycler gradient, 120V (Eppendorf, Hamburg, Germany)

Thermal cycling conditions consisted of 10 minutes at 25℃ followed by 30 minutes at 48℃ and 5 minutes at 95℃ with a hold stage on completion at 4℃. The resulting cDNA was frozen at -20℃ until use.

2.2.5 Analysis of mRNA expression by real-time qPCR

Following reverse transcription cDNA samples were prepared for real-time qPCR as follows; cDNA (40ng) was combined with qPCR master mix (4μM), sense and antisense primers and oligonucleotide probe mix (0.4μM) (Table 2.1). Separate reactions were also conducted with primers and probes for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control.

Thermal cycling conditions consisted of 15 minutes at 95℃ followed by 50 cycles of 15 seconds at 95℃ and 60℃. Samples were then held at 4℃. Quantification of PCR products occurred in real time and was analysed using the Chromo4 Real-Time Detection System (MJ Research, INC. Waltham, Massachusetts, USA). Expression data were normalised to GAPDH expression using the comparative ΔΔCt method consisting of 2 raised to the power of the difference in the Ct between the reference gene GAPDH and the test gene.

Table 2.1 TaqMan assay IDs for the assays of gene expression

Gene Symbol	TaqMan Assay ID
PXR	Hs00243666_m1
CAR	Hs00901571_m1
ABCB1	Hs01067802_m1
ABCC1	Hs00219905_m1
ABCC2	Hs00166123_m1
ABCC10	Hs00375716_m1
OATP1A2	Hs00366488_m1
OATP1B1	Hs00272374_m1
OATP1B3	Hs00251986_m1
OATP1C1	Hs00203184_m1
OATP2A1	Hs00213714_m1
OATP2B1	Hs00194554_m1
OATP3A1	Hs01030353_m1
OATP4A1	Hs00983994_m1
OATP4C1	Hs01030663_m1
OATP5A1	Hs00229597_m1
OATP6A1	Hs01053821_m1
GAPDH	Hs03929097_g1

2.2.6 Extraction of genomic DNA

20µl QIAGEN protease was pipetted into a sterile eppendorf (1.5ml microtube). 200µl whole blood and 200µl lysis buffer AL were added. The eppendorfs were then pulse-vortexed for 15s. The samples were incubated at 56℃ for 10 minutes, centrifuged at 6,000×g for 1 minute to remove any drops of sample from the lid. 200µl 100% ethanol was then added, the samples were pulse-vortexed for 15s and centrifuged at 6,000×g for 1 minute to remove any drops of sample from the lid.

All samples were carefully mixed and then transferred to a spin column without wetting the rim of the filter and centrifuged at 6,000×g for 1 minute. The filter was placed into a clean collection tube. 500µl wash buffer 1 was then added to the spin column, again without wetting the rim and was centrifuged at 6,000×g for 1 minute. The filter was placed into a clean collection tube.

500µl wash buffer 2 was then added to the spin column (avoiding wetting the rim) and the eppendorf was centrifuged at 13,000×g for 3 minutes to dry the membrane completely. The filtrate was discarded. If any remnants remain of buffer 2, it can inhibit downstream applications, e.g. PCR. In such case, further centrifugation was conducted using a clean collection tube to ensure no buffer carry-over. The filter was placed into a new sterile eppendorf.

50 µl elution buffer 3 was then carefully applied to the filter membrane. When extracting gDNA from patient samples, cell counts vary widely, hence eluting DNA in 50 µl elution buffer 3 is recommended to ensure sufficient concentrations.

The samples were incubated at room temperature for 3 minutes and centrifuged at 6,000 ×g for 1 minute to elute the gDNA. The filter was discarded and the DNA was stored at +4 ℃ for up to a month or stored long-term at -20 ℃.

2.2.7 Quantification of gDNA

25µl stock PicoGreen was diluted in 5ml ddH_2O. PicoGreen is light-sensitive so the diluted solution was stored in conditions protected from light. In a MJ white 96-well plate, 2µl of DNA sample was added to each well and 50µl of the diluted PicoGreen. The plate was covered and centrifuged briefly.

A standard curve was performed using DNA solution of known concentration in duplicate. DNA standards started at 200ng/µl serially diluted 1 in 2 down to 0ng/µl. A no-template control was included so background fluorescence could be taken into account.

The gDNA concentration was quantified by real-time PCR. The standard curve was found to follow a linear relationship ($Y = 0.221X + 0.0894$, $R^2 = 0.9805$) between concentration and RFU (Figure 2.1).

The average fluorescence was calculated for each standard and used to plot a scatter graph (Figure 2.1). There should be 6 standard concentrations used. A straight line of best fit was added and using the equation of the line, the DNA concentrations of unknown samples were calculated.

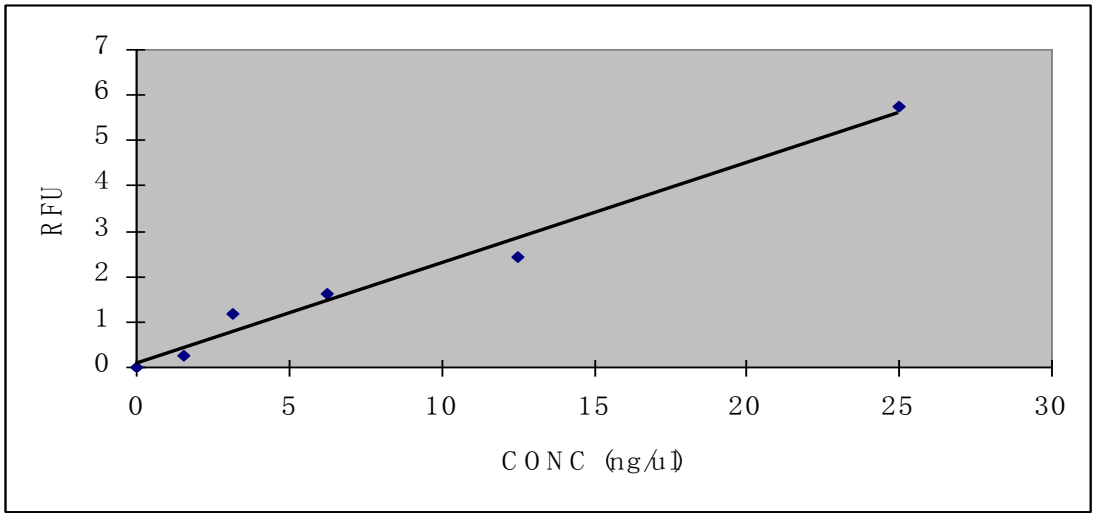


Figure 2.1 Standard curve showing relationship between concentration and RFU

2.2.8 Nuclear receptor polymorphisms genotyping

Genomic DNA concentrations were normalized to 20ng/µl. Samples were prepared for real-time qPCR as follows: 20×Primer mix (1.25µl 1.8µM final concentration), 20×Probe mix (1.25µl 0.4µM final concentration) 2×qPCR Master Mix (12.5µl) and gDNA (2µl) (Table 2.2). Reaction mixtures were then made up to 25µl final volume using DNase free water.

Thermal cycling conditions consisted of 15 minutes at 95℃ followed by 50 cycles of 15 seconds at 95℃ and 1 minute at 60℃. Samples were then held at 4℃. Quantification of PCR products occurred in real-time and was analysed using the Chromo4 Real-Time Detection System (MJ Research, INC. Waltham, Massachusetts, USA).

Table 2.2 TaqMan assay IDs for the assays of genotype

SNPs	TaqMan Assay ID
PXR44477 T>C	C_9152783_20
PXR63396 C>T	C_26079845_10
PXR69789 A>G	C_1834250_10
CAR540 C>T	C_25746794_20

2.2.9 Statistical analysis

The gene expression of drug transporters and nuclear receptors were log10 transformed and given as median (range) unless stated otherwise. Distribution of the gene expression of drug transporters was determined via Shapiro-Wilk test. The relationships with gene expression of drug transporters were assessed by Mann-Whitney test (for genotype groups) or by simple linear regression (continuous data such as the gene expression of nuclear receptors) using Pearson's rank correlation coefficient. Rho is a non-parametric correlation coefficient. A p-value < 0.05 was considered to identify groups that were significantly different from each other and a p-value < 0.1 was used as a cut off for trend. The genotypes with rare double variants (defined by < 3 in the total sample population) were treated as one genotype with the heterozygous variants in these analyses. These statistical analyses were conducted using Stats Direct (Stats Direct Ltd., Cheshire, UK)

Multivariate analysis was conducted to construct a predictive model using patient demographic, nuclear receptor gene expression and genotypes as independent variables and the gene expression of drug transporters as the dependent variable. Dichotomous variables, including gender, were coded as 0 for female and 1 for male, while *PXR44477*, *PXR63396*, *PXR69789* and *CAR540* genotypes were coded as 0, 1 and 2 for homozygous wild type, heterozygous variants and homozygous variants, respectively. Ethnicity was also coded as 0, 1 and 2 for White British, Black African and others respectively, while age was included as a continuous variable. Univariate

analysis was used to select independent variables with p-values < 0.2 to be included in the multivariate analysis. These statistical analysis were conducted using SPSS 20 (SPSS Inc., Chicago, IL, USA)

2.3 RESULTS

2.3.1 Patient demographics

84 patients were recruited from the Royal Liverpool University Hospital. Patient characteristics, including age, gender and ethnicity, were obtained from medical records. The group comprised with 56% males and 43% females (one person no record). The average age of patients was 60.3 years old with a range of 17 to 88 years old. The ethnicity included 89.3% White British, 3.6% African and 7.2% others (Table 2.3).

Table 2.3 demographics of patient

Demographics		
Age (yrs)	Average	60.3 ± 17.6
	Range	17 to 88
Gender (n, %)	Male	47，56
	Female	36，43
	No record	1, 1
Ethnicity (n, %)	White British	75，89.3
	Black African	3，3.6
	others	6，7.2

2.3.2 Correlation of nuclear receptors and ABC transporters expression

The correlation of gene expression in intestine between nuclear receptors (PXR and CAR) and ABC transporters (ABCB1, ABCC1, ABCC2 and ABCC10) were analysed. Relationships between PXR & ABCB1 (Rho = 0.46, $P < 0.0001$), PXR & ABCC1 (Rho = 0.52, $P < 0.0001$), PXR & ABCC2 (Rho = 0.52, $P < 0.0001$) and PXR & ABCC10 (Rho = 0.71, $P < 0.0001$) were evident (Figure 2.2). There were also correlations between CAR & ABCB1 (Rho = 0.41, $P = 0.0004$) and CAR & ABCC2 (Rho = 0.44, $P = 0.0002$) (Figure 2.3).

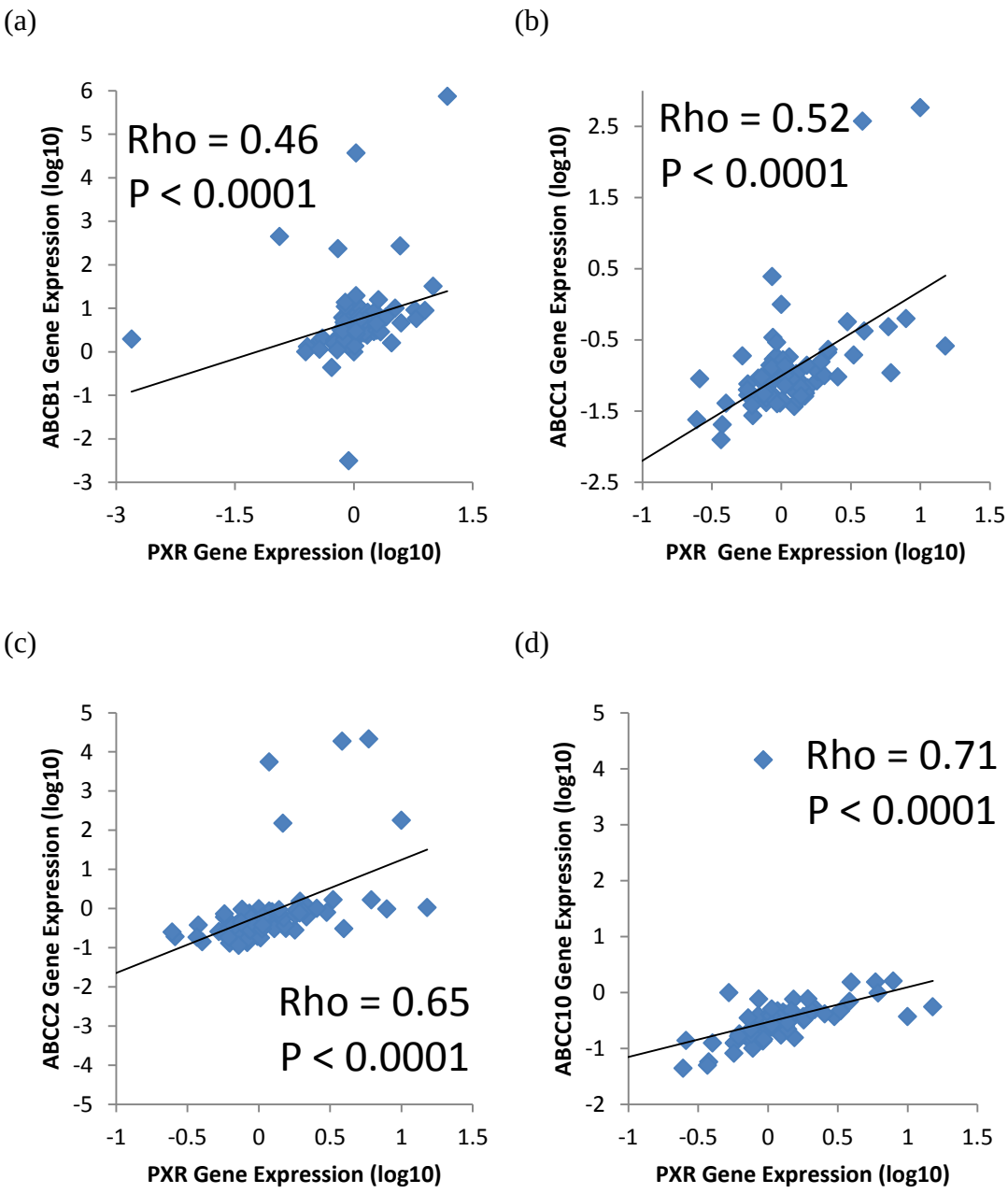


Figure 2.2 Correlation of gene expression between PXR and ABC transporters in intestine. Correlation between the gene expression of (a) PXR & ABCB1, (b) PXR & ABCC1, (c) PXR & ABCC2 and (d) PXR & ABCC10

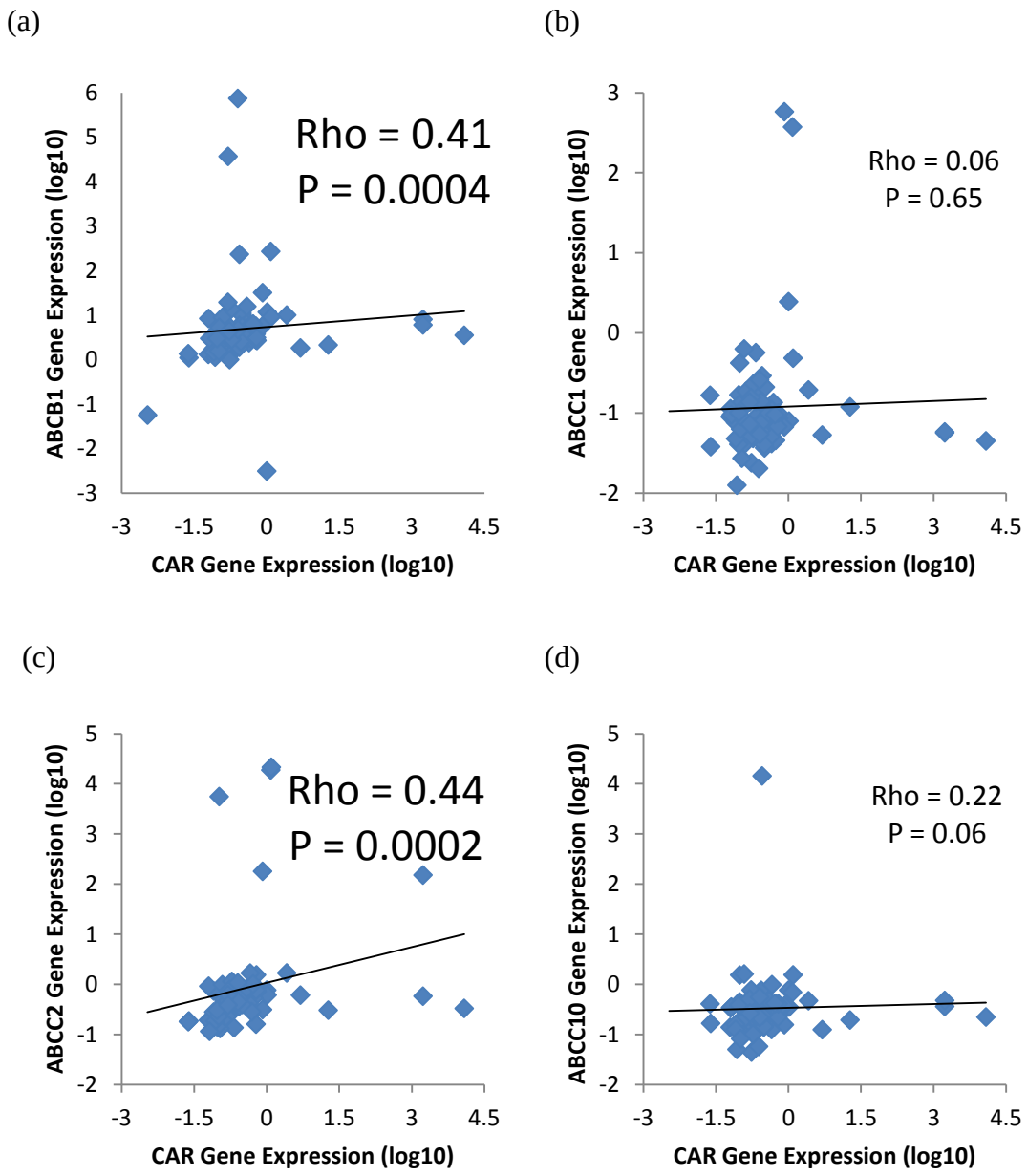


Figure 2.3 Correlation of gene expression between CAR and ABC transporters in intestine. Correlation between the gene expression of (a) CAR & ABCB1, (b) CAR & ABCC1, (c) CAR & ABCC2 and (d) CAR & ABCC10

2.3.3 Correlation of nuclear receptors and OATP transporters expression

Only some biopsy samples had detectable expression of OATP transporters (Table 2.4). Over 50 % of the intestinal biopsies contained detectable levels of specific mRNA for 3 transporters (OATP2B1, 2A1 and 3A1). OATP2B1 was the most frequently detected, with 83% of the samples showing expression, with an average gene expression of 3.63. OATP2A1 was expressed in 80% of biopsies and had an average gene expression of 1.8. For OATP3A1, 60% of biopsy samples expressed it with an average gene expression of 3.1. Although the number of detectable samples for OATP4A1, OATP4C1 and OATP5A1 was lower than 50%, they were still expressed in some biopsy samples. OATP4A1 and OATP5A1 were expressed in 38% and OATP4C1 in 46% of the biopsies. The average of their expression was 30.3, 5.9 and 12.7. However, for the other transporters, less than 1% of the biopsies expressed them with no biopsies expressing OATP6A1. From Figure 2.4, it is clear that there is high variability in these OATP transporters. In conclusion, OATP2A1, OATP2B1, OATP3A1, OATP4A1, OATP4C1 and OATP5A1 are expressed in intestine and there was a high variability in these OATP transporters.

Therefore, the correlation of gene expression in intestine between nuclear receptors (PXR and CAR) and OATP transporters (OATP2A1, OATP2B1, OATP3A1, OATP4A1, OATP4C1 and OATP5A1) were analysed. Of particular interest, PXR & OATP2A1 (Rho = 0.56, $P < 0.0001$) and PXR & OATP2B1 (Rho = 0.39, $P = 0.001$) gene expression in intestine were positively correlated (Figure 2.5). Other relationships emerged between CAR and OATP4C1 (Rho = 0.40, $P = 0.01$) (Figure 2.6). These correlations are summarised in Table 2.5

Table 2.4 Detectable expression of OATP transporters in intestine

Genes	Samples amount with detectable mRNA (%)	Mean (range) expression (ΔΔCt) in detectable samples
OATP1A2	4.9%	1231.3 (0.04, 4921.9)
OATP1B1	6.2%	0.4 (0.0009, 1)
OATP1B3	7.4%	0.2 (1.7E-06, 1)
OATP1C1	3.70%	0.8 (0.1, 1.4)
OATP2A1	80.2%	1.8 (0.01, 45.6)
OATP2B1	82.7%	3.6 (0.008, 186.1)
OATP3A1	60.5%	3.1 (0.09, 64.4)
OATP4A1	38.3%	30.3 (0.5, 823.1)
OATP4C1	46.9%	12.7 (0.4, 123.6)
OATP5A1	38.3%	5.9 (0.4, 84.4)
OATP6A1	0	0

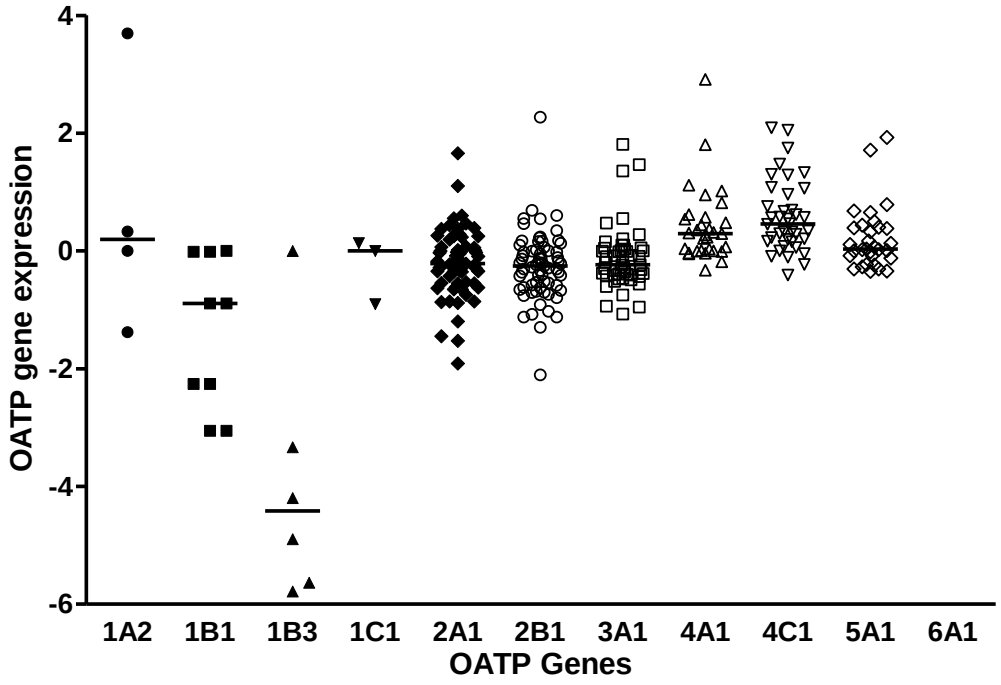
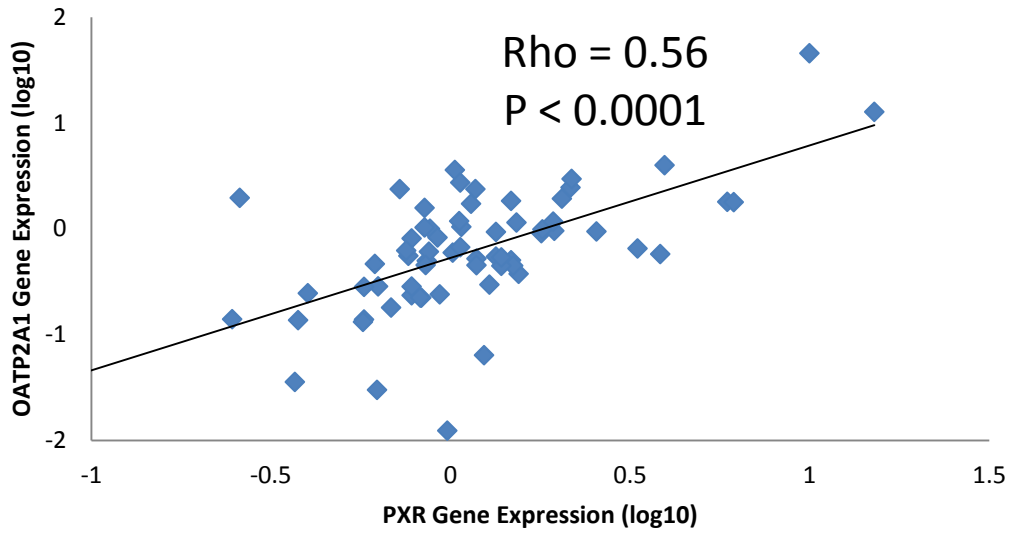


Figure 2.4 Gene expressions of OATP transporters in intestine

(a)



(b)

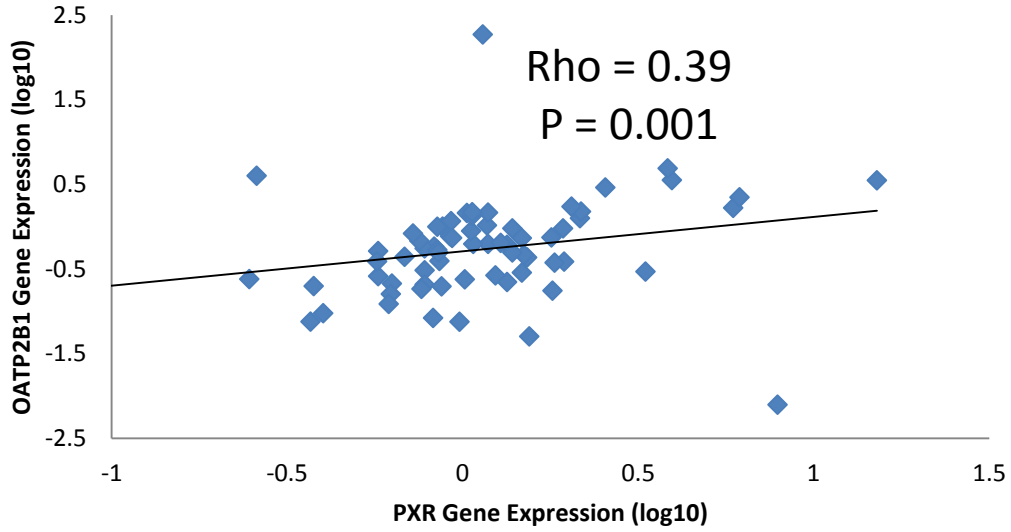


Figure 2.5 Correlation of gene expression between PXR and OATP transporters in intestine. Correlation between the gene expression of (a) PXR & OATP2A1 and (b) PXR & OATP2B1

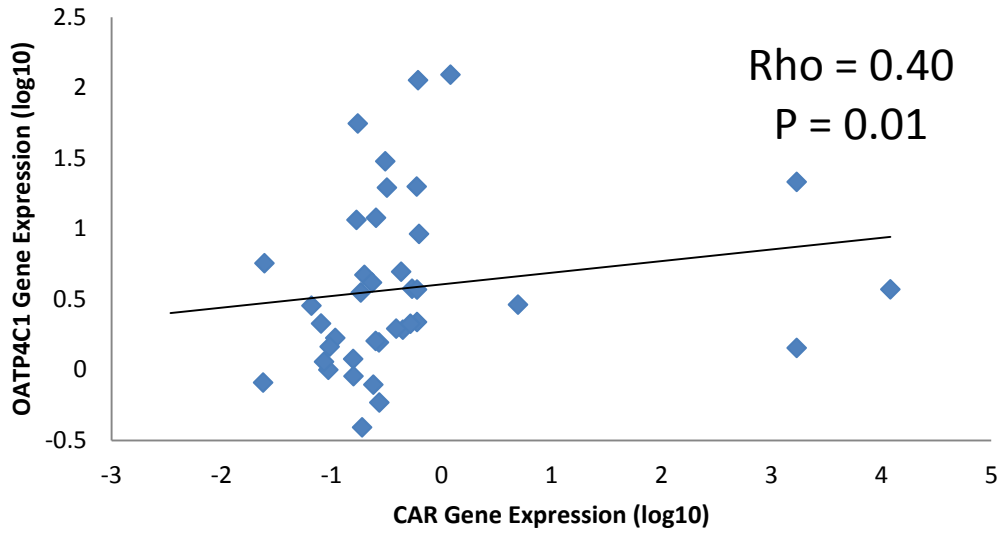


Figure 2.6 Correlation of gene expression between CAR and OATP transporters in intestine. Correlation between the gene expression of CAR and OATP4C1

Table 2.5 Correlation of gene expression between nuclear receptors and OATP transporters in intestine

GENES	PXR		CAR	
	P value	**Rho**	**P value**	**Rho**
OATP2A1	**< 0.0001**	**0.56**	0.39	0.11
OATP2B1	**0.001**	**0.39**	0.85	0.02
OATP3A1	0.12	0.22	0.06	0.27
OATP4A1	0.49	0.13	0.59	0.10
OATP4C1	0.10	0.27	**0.01**	**0.40**
OATP5A1	0.73	0.06	0.28	-0.20

2.3.4 Allele frequencies for PXR and CAR polymorphisms

Three *PXR* polymorphisms (*PXR44477*, *PXR69789* and *PXR63396*) and one CAR (*CAR540*) polymorphism were investigated in the 84 patients. Allele frequencies for the *PXR44477* polymorphism were 0.51 for the C allele and 0.49 for the T allele (Table 2.6). The allele frequencies for *PXR63396* polymorphism were 0.52 for the T allele and 0.48 for the C allele (Table 2.6). The *PXR69789* polymorphism allele frequencies were 0.40 for G allele and 0.60 for the A allele (Table 2.6). The *CAR540* polymorphism was found at a frequency of 0.34 for the T allele and 0.66 for the C allele (Table 2.6). The observed genotype frequency was in Hardy-Weinberg equilibrium.

Table 2.6 Frequencies of PXR and CAR polymorphisms

SNPs	**Genotype Frequency n (%)**			**Allele Frequency %**	
PXR44477	**CC**	**CT**	**TT**	**C**	**T**
T>C	22 (28.2)	36 (46.2)	20 (25.6)	51.3	48.7
PXR63396	**TT**	**CT**	**CC**	**T**	**C**
C>T	20 (25.6)	41 (52.6)	17 (21.8)	51.9	48.1
PXR69789	**GG**	**GA**	**AA**	**G**	**A**
A>G	12 (15.4)	39 (50.0)	27 (34.6)	40.4	59.6
CAR540	**TT**	**CT**	**CC**	**T**	**C**
C>T	8 (10.3)	37 (47.4)	33 (42.3)	34.0	66.0

2.3.5 Impact of nuclear receptor SNPs on drug transporters expression

2.3.5.1 Impact of PXR44477 SNP on drug transporters expression

There was a significant association between *PXR44477* polymorphism and ABCC10 gene expression (P = 0.021) in intestine (Figure 2.7). No significant association was found between *PXR44477* polymorphism and gene expression of other ABC transporters (ABCB1, ABCC1 and ABCC2) and OATP transporters (OATP2A1, OATP2B1, OATP3A1, OATP4A1, OATP4C1 and OATP5A1) in intestine (Figure 2.7).

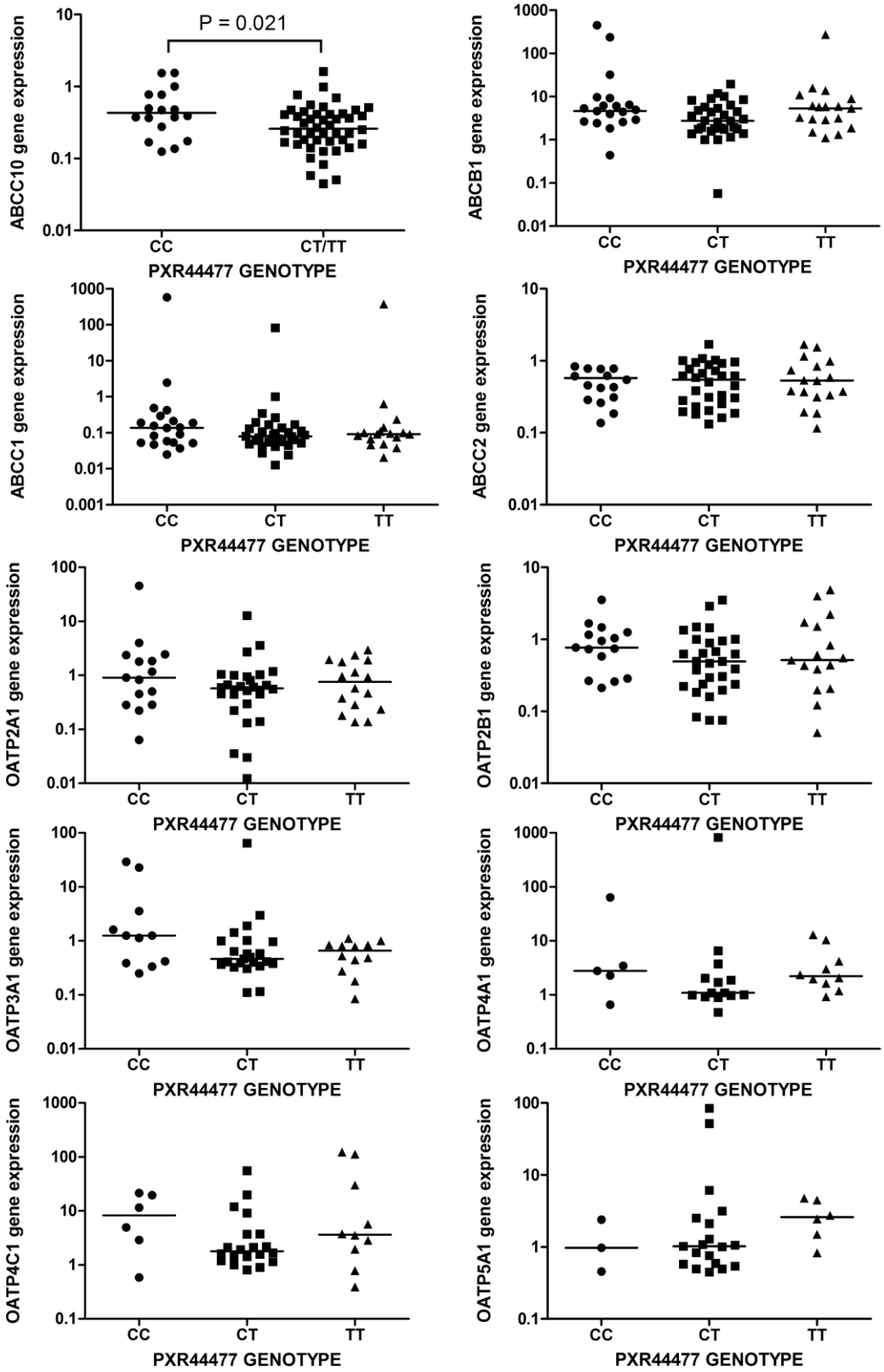


Figure 2.7 Impact of *PXR44477* SNP on drug transporters expression. The lines indicate the median values.

2.3.5.2 Impact of *PXR63396* SNP on drug transporters expression

PXR63396 polymorphism was associated with ABCB1 gene expression (P = 0.0496) and ABCC2 gene expression (P = 0.023) in intestine (Figure 2.8). No other significant associations were found between *PXR63396* polymorphism and gene expression of ABC transporters (ABCC1 and ABCC10) and OATP transporters (OATP2A1, OATP2B1, OATP3A1, OATP4A1, OATP4C1 and OATP5A1) in intestine (Figure 2.8).

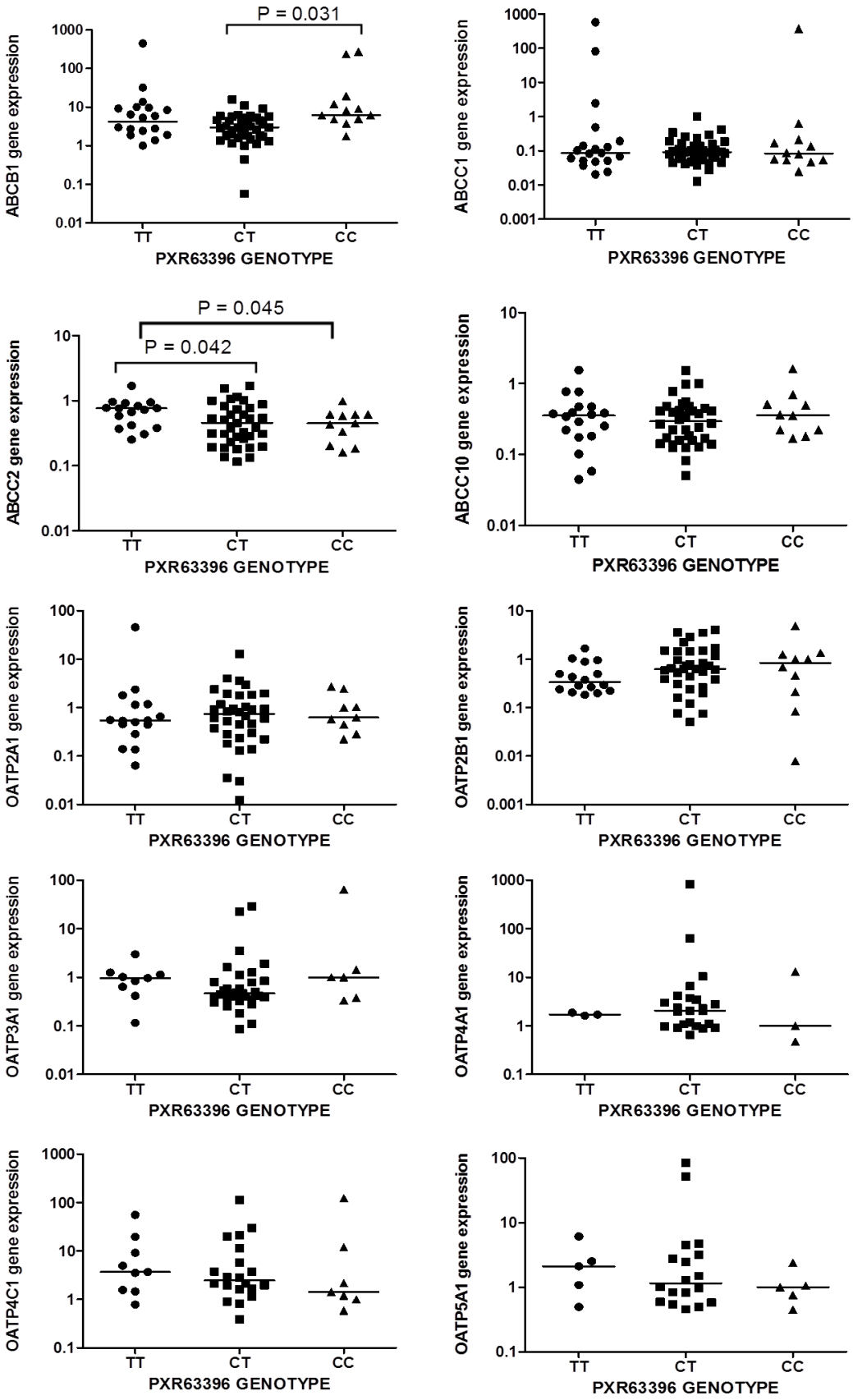


Figure 2.8 Impact of *PXR63396* SNP on drug transporters expression. The lines indicate the median values.

2.3.5.3 Impact of *PXR69789* SNP on drug transporters expression

No significant associations were found between *PXR69789* polymorphism and gene expression of ABC transporters (ABCB1 and ABCC1) and OATP transporters (OATP2A1, OATP2B1, OATP3A1, OATP4A1, OATP4C1 and OATP5A1) in intestine (Figure 2.9).

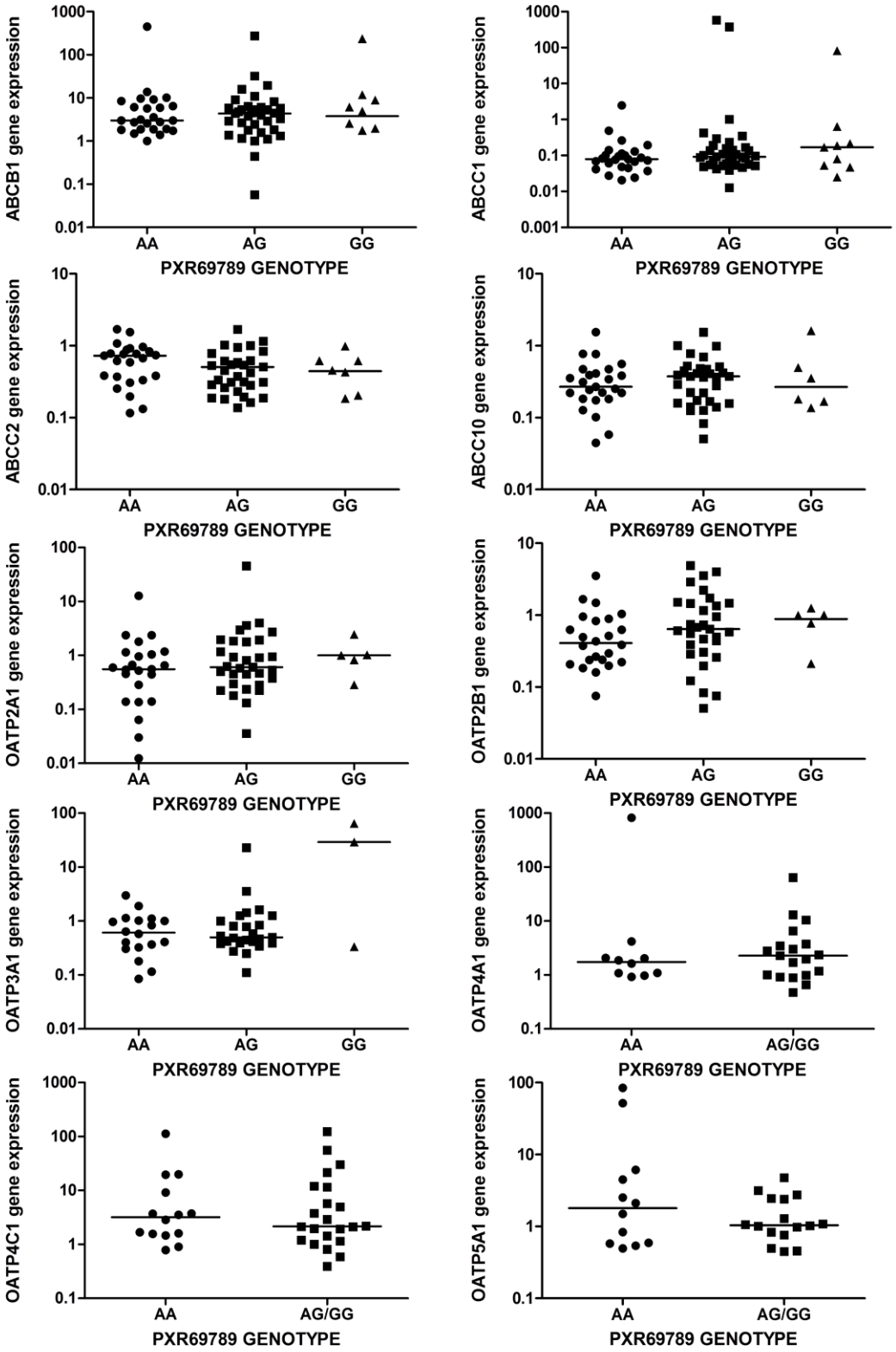


Figure 2.9 Impact of *PXR69789* SNP on drug transporters expression. The lines indicate the median values.

2.3.5.4 Impact of *CAR540* on drug transporters expression

There were no significant associations between *CAR540* polymorphism and gene expression of ABC transporters (ABCB1, ABCC1, ABCC2 and ABCC10) or OATP transporters (OATP2A1, OATP2B1, OATP3A1, OATP4A1, OATP4C1 and OATP5A1) in intestine (Figure 2.10).

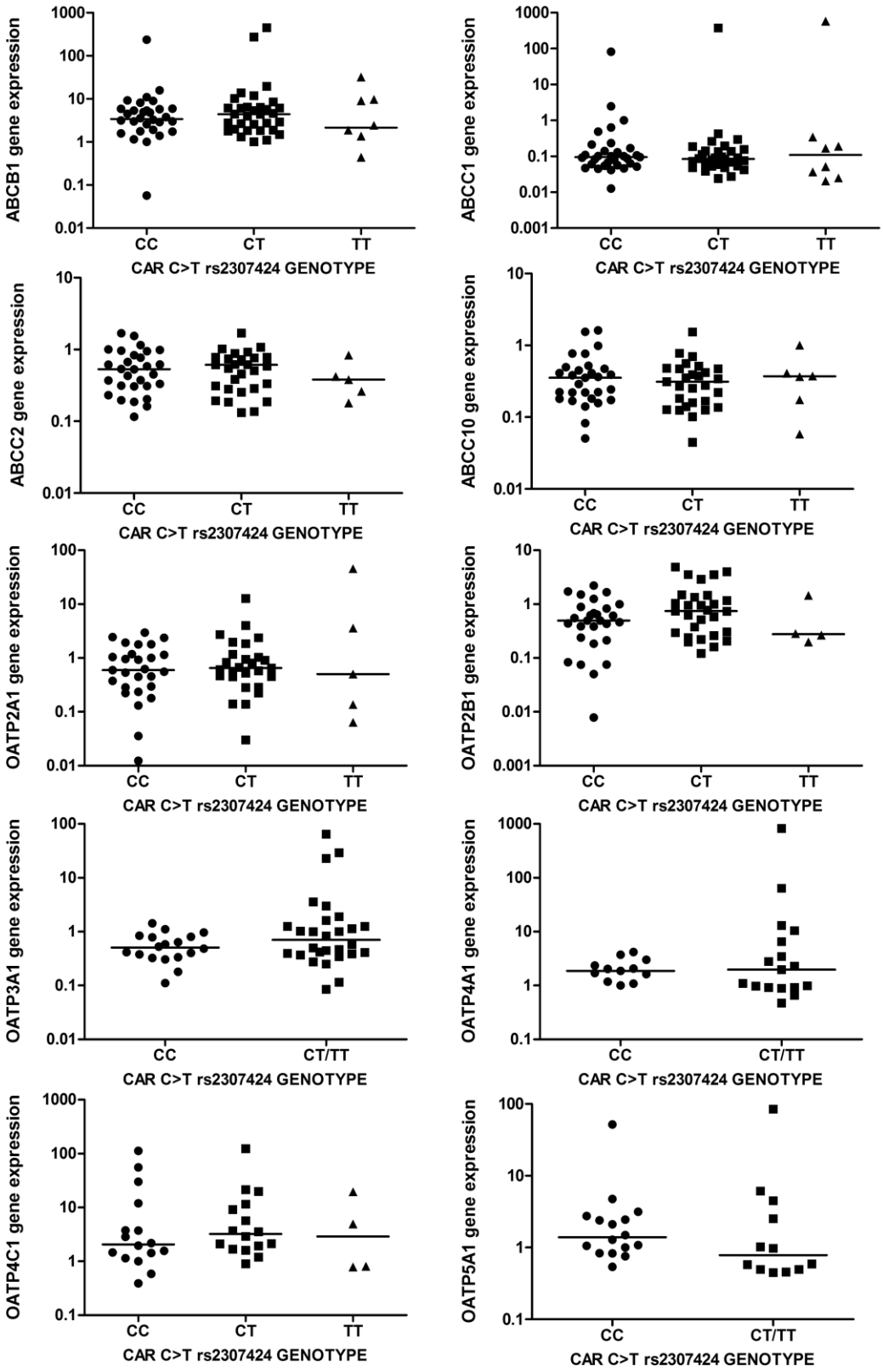


Figure 2.10 Impact of *CAR540* on drug transporters expression. The lines indicate the median values.

2.3.6 Multivariate analysis of drug transporters expression

2.3.6.1 Patient characteristics and nuclear receptor expression

Using multiple linear regression, associations between the expression of drug transporters with patient characteristics including age, gender, ethnicity and nuclear receptor (PXR and CAR) gene expression in intestine were analysed and are summarised in Table 2.7 and 2.8. In univariate analysis, the gene expression of ABCB1, ABCC1, ABCC2, OATP2A1, OATP2B1 and OATP4A1 were significantly associated with PXR gene expression. Meanwhile, the gene expression of these transporters (except ABCC2) also had a significant association with PXR gene expression in multivariate analysis. However, a trend towards association was found between ABCC2 and PXR gene expression in multivariate analysis.

Table 2.7 Univariate and multivariate linear regression analysis of ABC transporters gene expression with patient characteristics and nuclear receptor gene expressions

Covariate	ABCB1 gene expression Univariate		ABCB1 gene expression Multivariate	
	β	**P**	**β**	**P**
Age	451 (-741 to 1644)	0.45	-	-
Gender	17356 (-23487 to 58199)	0.40	-	-
Ethnicity	-5339 (-50101 to 39423)	0.81	-	-
CAR gene expression	-1 (-15 to 13)	0.88	-	-
PXR gene expression	25717 (19322 to 32113)	**$1.4x10^{-11}$**	25717 (19322 to 32113)	**$1.4x10^{-11}$**
Covariate	**ABCC1 gene expression Univariate**		**ABCC1 gene expression Multivariate**	
	β	**P**	**β**	**P**
Age	0.081 (-1 to 1)	0.88	-	-
Gender	-29 (-66 to 8)	0.13	-24 (-59 to 10)	0.17
Ethnicity	-7 (-49 to 34)	0.72	-	-
CAR gene expression	-0.001 (-0.015 to 0.012)	0.83	-	-
PXR gene expression	14 (7 to 22)	**0.0003**	13 (6 to 21)	**0.0004**

Table 2.8 Univariate and multivariate linear regression analysis of OATP transporters gene expression with patient characteristics and nuclear receptor gene expressions

Covariate	OATP2A1 gene expression Univariate		OATP2A1 gene expression Multivariate	
	β	**P**	**β**	**P**
Age	0.06 (-0.02 to 0.1)	0.14	0.03 (-0.03 to 0.09)	0.34
Gender	-1.2 (-3.7 to 1.4)	0.37	-	-
Ethnicity	-0.5 (-3.3 to 2.3)	0.73	-	-
CAR gene expression	-0.00008 (-0.001 to 0.001)	0.86	-	-
PXR gene expression	1.4 (1.0 to 1.9)	**6.8×10^{-9}**	1.4 (0.1 to 1.9)	**1.6×10^{-8}**
Covariate	**OATP2B1 gene expression Univariate**		**OATP2B1 gene expression Multivariate**	
	β	**P**	**β**	**P**
Age	0.081 (-1 to 1)	0.88	-	-
Gender	-29 (-66 to 8)	0.13	-24 (-59 to 10)	0.17
Ethnicity	-7 (-49 to 34)	0.72	-	-
CAR gene expression	-0.001 (-0.015 to 0.012)	0.83	-	-
PXR gene expression	14 (7 to 22)	**0.0003**	13 (6 to 21)	**0.0004**
Covariate	**OATP4A1 gene expression Univariate**		**OATP4A1 gene expression Multivariate**	
	β	**P**	**β**	**P**
Age	0.002 (-0.01 to 0.02)	0.77	-	-
Gender	-0.08 (-0.5 to 0.4)	0.75	-	-
Ethnicity	-0.2 (-0.7 to 0.3)	0.53	-	-
CAR gene expression	-0.00004 (-0.0002 to 0.0001)	0.58	-	-
PXR gene expression	0.2 (0.09 to 0.3)	**0.0002**	0.2 (0.09 to 0.3)	**0.0002**

2.3.6.2 Patient characteristics and nuclear receptor polymorphisms

Associations between the gene expression of drug transporters with patient characteristics and nuclear receptor polymorphisms in intestine were analysed by using multiple linear regression, which are summarised in Table 2.9 and 2.10. ABCC1 gene expression had a trend towards association with *CAR540* polymorphism in univariate analysis, while it was also significant associated with *CAR540* polymorphism in multivariate analysis (Table 2.9). OATP2A1 gene expression was significantly associated with *CAR540* polymorphism in both univariate and multivariate analysis (Table 2.10). OATP3A1 gene expression was found to be significantly associated in univariate analysis and a trend towards association in multivariate analysis with *PXR69789* polymorphism. In univariate analysis, OATP4C1 gene expression had a trend towards association with *PXR44477* polymorphism, while it not only had a significant association with *PXR44477* polymorphism but also a trend towards association with patients' age in multivariate analysis (Table 2.10). A trend towards association was observed between OATP5A1 gene expression and *PXR69789* polymorphism in both univariate and multivariate analysis (Table 2.10).

Table 2.9 Univariate and multivariate linear regression analysis of ABCC1 gene expression with patient characteristics and nuclear receptor polymorphisms

Covariate	ABCC1 gene expression Univariate		ABCC1 gene expression Multivariate	
	β	**P**	**β**	**P**
Age	-0.08 (-1 to 1)	0.88	-29 (-66 to 7)	0.12
Gender	-29 (-66 to 8)	0.13	-	-
Ethnicity	-7 (-49 to 34)	0.72	-	-
CAR540	28 (-0.4 to 56)	**0.053**	28 (0.04 to 56)	**0.0497**
PXR44477	5 (-21 to 31)	0.72	-	-
PXR63396	-7 (-35 to 21)	0.62	-	-
PXR69789	10 (-18 to 37)	0.49	-	-

Table 2.10 Univariate and multivariate linear regression analysis of OATP transporters gene expression with patient characteristics and nuclear receptor polymorphisms

Covariate	OATP2A1 gene expression Univariate		OATP2A1 gene expression Multivariate	
	β	**P**	**β**	**P**
Age	0.06 (-0.02 to 0.1)	0.14	0.05 (-0.02 to 0.1)	0.17
Gender	-1 (-4 to 1)	0.37	-	-
Ethnicity	-0.5 (-3 to 2)	0.73	-	-
CAR540	2 (0.2 to 4)	**0.03**	2 (0.1 to 4)	**0.04**
PXR44477	1 (-0.7 to 3)	0.25	-	-
PXR63396	-1 (-3 to 0.8)	0.26	-	-
PXR69789	0.07 (-2 to 2)	0.94	-	-
Covariate	**OATP4C1 gene expression Univariate**		**OATP4C1 gene expression Multivariate**	
	β	**P**	**β**	**P**
Age	-0.2 (-0.5 to 0.08)	0.16	-0.3 (-0.5 to 0.03)	0.07
Gender	-3 (-13 to 7)	0.52	-	-
Ethnicity	-1 (-12 to 10)	0.85	-	-
CAR540	-1 (-9 to 6)	0.72	-	-
PXR44477	-6 (-13 to 0.3)	0.06	-7 (-14 to -0.7)	**0.03**
PXR63396	2 (-5 to 9)	0.58	-	-
PXR69789	-2 (-9 to 5)	0.54	-	-

2.4 DISCUSSION

Nuclear receptors are recognised to be involved in the transcriptional regulation of ABC transporters and OATP transporters. In 2005, Albermann *et al.* found all transporters including ABCB1, ABCC1 and ABCC2 had a positive correlation with PXR expression in PBMCs and intestine (except ABCC1 in the intestine) but no relationship between PXR expression and the expression of ABCB1, ABCC1 and ABCC2 in liver (Albermann *et al.*, 2005). In addition, PXR and CAR increased the ABCB1 transcription and ABCC2 was also regulated by PXR in human intestinal epithelial cells (Haslam *et al.*, 2008). Recently, Jeannesson *et al* showed the expression of ABCB1 and PXR were not significantly correlated in PBMCs (Jeannesson *et al.*, 2011). In this study, we found a positive correlation between PXR expression and the expression of ABCB1, ABCC1, ABCC2 and ABCC10 in intestine. However, the data for ABCC1 is not in agreement with the previous study (Albermann *et al.*, 2005). Our data also show correlations of expression between CAR and these ABC transporters (except ABCC1) in intestine. These data agree with the ABCB1 study of Haslam *et al* in intestine. We also extended this analysis to ABCC10, and relationships were also found between PXR and CAR gene expression with ABCC10 gene expression in intestine.

Our data show that OATP2A1, OATP2B1, OATP3A1, OATP4A1, OATP4C1, and OATP5A1 transporters are expressed in intestine. However, there is a high variability in these OATP transporters. Part of the reason for this variability may be the precise location of the intestine from which the biopsies were taken. Nevertheless, it is likely that pharmacogenetic and/or environmental factors also contribute to this variability. In the literature, OATPs are expressed in various cells and tissues, some members show a more restricted pattern (well-studied OATP1B1/OATP1B3 in liver, OATP4C1 in kidney, and OATP6A1 in testis) (Svoboda *et al.*, 2011). Only OATP2B1 has been found to be expressed in the intestine and this is in agreement with our data (Visentin *et al.*, 2011). However, in contrast to our results, previous studies have shown that OATP1A2 is expressed in the intestine (Tamai, 2011). There were significant relationships between the expression of CAR with OATP3A1 and CAR with OATP4C1 in intestine. Also, it is likely that PXR expression is associated with the gene expression of OATP2A1 and OATP2B1.

This chapter also investigated associations between nuclear receptor (PXR and CAR) polymorphisms and the gene expression of drug transporters. A statistically significant association emerged between *PXR63396* polymorphism and the expression of ABCB1 and ABCC2 in intestine, while *PXR44477* polymorphism was associated with ABCC10 expression in intestine. *PXR63396* alters PXR expression and CYP3A4 activity *in vitro* and there is an association of *PXR63396* polymorphism with reduced concentrations of unboosted Atazanavir (Schipani *et al.*, 2010). *PXR* (rs2276707)

polymorphism was weakly associated with ulcerative colitis susceptibility (Glas *et al.*, 2011) and *PXR25385 (C>T)* polymorphism was identified as a significant covariate for apparent oral clearance of tacrolimus (Benkali *et al.*, 2009). PXR promoter and intron 1 SNPs associated with PXR target gene expression (CYP3A4) in donor livers and cultured hepatocytes has also been reported (Lamba *et al.*, 2008).

Multivariate linear regression analysis revealed that PXR expression appeared to be involved in the expression of ABC transporters (ABCB1, ABCC1 and ABCC2) and OATP transporters (OATP2A1, OATP2B1 and OATP4A1). PXR gene expression significantly influenced the gene expression of ABCB1, ABCC1, ABCC2, OATP2A1, OATP2B1 and OATP4A1. CAR polymorphism significantly affected the gene expression of ABCC1 and OATP2A1, while *PXR69789* polymorphism had a trend to associate with OATP3A1 and OATP5A1 gene expression. In addition, OATP4C1 was significantly influenced by *PXR44477* polymorphism and had a trend to associate with the age of patients.

In summary we have shown the impact of nuclear receptor gene expression on the expression of drug influx and efflux transporters. Also, this chapter describes novel associations between nuclear receptor polymorphisms and drug transporter gene expression.

CHAPTER 3

Impact of nuclear receptors expression and single nucleotide polymorphisms on the gene expression of cytochrome P450 enzymes in intestine

Contents

3.1 INTRODUCTION

In chapter 2, we investigated the impact of PXR and CAR in the expression of ABC transporters which play an important role on influencing the pharmacokinetics of orally dosed drugs (Fromm, 2000; Landowski *et al.*, 2003; Mizuno *et al.*, 2002). However, enzymes such as Cytochrome P450 are also involved in drug metabolism and bioactivation (Wang *et al.*, 2010). Previous study showed that transporter and enzyme played complementary roles in drug absorption, distribution, metabolism and excretion by biotransformation and counter-transport, particularly in the intestine (Benet, 2009). Literature evidence indicates that the positive correlation between PXR and ABC transporters in intestine is reversed in liver – this would be consistent with enabling increased hepatic residence time and increased metabolism in liver，and in intestine increased presentation of drug to CYP3A4 and more metabolisms as a result of cycles of efflux. Due to increase ABC transporter expression in intestine, there is no need to change CYP3A4 expression level.

In this study, we focus on impact of nuclear receptors (PXR and CAR) on the expression of Cytochrome P450 enzymes including CYP2B6 and CYP3A4 in intestine. In literature, the human nuclear receptors PXR and CAR are known to regulate gene expression of the Cytochrome P450 enzymes, especially, CYP2B6 and CYP3A4 (Rhodes *et al.*, 2011). CAR and PXR expression correlate with CYP2B6 gene expression in liver (Chang *et al.*, 2003). The mRNA expression of CYP3A is

also regulated by several nuclear receptors such as PXR and CAR (Hasegawa *et al.*, 2010).

A number of pharmacological factors influence drug metabolism, including genetic variability in metabolizing enzymes and their regulators which possess the capacity to modulate enzyme activity and/or expression. These factors become particularly important when complex drug regimens are used as is the case in HIV treatment. The CYP2B6 polymorphisms are associated with altered hepatic CYP2B6 expression and activity (Wyen *et al.*, 2011). CYP2B6 genotype influences plasma efavirenz and nevirapine concentrations (Uttayamakul *et al.*, 2010). However, in other studies, CYP2B6 polymorphisms were associated with high plasma efavirenz concentrations but not nevirapine, while *CYP3A4* polymorphisms had no significant impact on plasma efavirenz or nevirapine concentrations in a Chinese patient population (Chen *et al.*, 2010).

As PXR regulates Cytochrome P450 enzymes, the PXR polymorphisms have revealed not only changes in PXR expression and activity, but also effects on CYP3A4 gene expression (Svard *et al.*, 2010). PXR polymorphism was associated with significantly lower expression of CYP3A4 in liver tissues (Sandanaraj *et al.*, 2008). Furthermore, in antiretroviral therapy, several drugs including darunavir, fosamprenavir, lopinavir, nelfinavir, tipranavir, efavirenz, and abacavir increased the expression of Cytochrome P450 enzymes through PXR (Svard *et al.*, 2010).

Chapter 2 detailed the impact of nuclear receptor expression and single nucleotide polymorphism on drug transporters in intestine. The aim of this chapter was to investigate the correlation between the expression of nuclear receptor (PXR and CAR) and Cytochrome P450 enzymes (CYP2B6 and CYP3A4) in intestine. The impact of single nucleotide polymorphism of PXR (*PXR*44477 *T>C* [rs1523130], *PXR*63396 *C>T* [rs2472677] and *PXR*69789 *A>G* [rs7643645]), CAR (*CAR540 C>T* [rs2307424]), CYP2B6 (*CYP2B6 516 G>T* [rs3745274] and *CYP2B6 1459 C>T* [rs3211371]) and CYP3A4 (*CYP3A4 392 A>G* [rs2740574]) on the gene expression of Cytochrome P450 enzymes in intestine was also investigated.

3.2 METHODS

3.2.1 Materials

TRIzol reagent, chloroform and nuclease free water were purchased from Sigma-Aldrich (Poole, UK). Isopropyl alcohol and ethanol were obtained from Fisher Scientific (Loughborough, UK). TaqMan reverse transcription (RT) kits were purchased from Applied Biosystems (Warrington, UK). Primers and probes for real-time PCR based gene expression assay and allelic discrimination assay were obtained from Applied Biosystems (Warrington, UK). qPCR master mix was obtained from ABgene (Epsom, UK). Whole blood DNA extraction kit purchased from QIAGEN (West Sussex, UK).

3.2.2 Patients Data

84 patients were recruited from the Royal Liverpool University Hospital. Patient characteristics, including sex, age and ethnicity, were obtained from medical records. The group comprised 47 male and 36 female patients (one person no record). The average age was 60.3 years old with a range of 17 to 88 years old. The ethnicity of these patients included White British, African and others. The intestinal biopsy samples from duodenum were used to investigate the human gene expression and the matched blood samples were used for genotype. Insufficient biopsy material was available to quantify protein expression.

3.2.3 Extraction and Reverse Transcription of mRNA

The mRNA samples were extracted according to the manufacturer's instructions as outlined in section 2.2.3. The cDNA samples were generated form mRNA by reverse transcription according to section 2.2.4. The cDNA concentrations were normalised to 20ng/µl.

3.2.4 Analysis of mRNA by real-time qPCR

Following reverse transcription cDNA samples were prepared for real-time qPCR as follows; cDNA (40ng) was combined with qPCR master mix (4μM), sense and antisense primers and oligonucleotide probe mix (0.4μM) (Table 3.1). Separate reactions were also conducted with primers and probes for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control.

Thermal cycling conditions consisted of 15 minutes at 95℃ followed by 50 cycles of 15 seconds at 95℃ and 60℃. Samples were then held at 4℃. Quantification of PCR products occurred in real time and was analysed using the Chromo4 Real-Time Detection System (MJ Research, INC. Waltham, Massachusetts, USA). Expression data were normalised to GAPDH expression using the comparative ΔΔCt method consisting of 2 raised to the power of the difference in the Ct between the reference GAPDH and the test gene.

Table 3.1 TaqMan assay IDs for the assays of gene expression

Genes	TaqMan Assay ID
PXR	Hs00243666_m1
CAR	Hs00901571_m1
CYP2B6	Hs03044636_m1
CYP3A4	Hs00430021_m1

3.2.5 Extraction of genomic DNA

Genomic DNA was extracted according to the manufacturer's instruction as outlined in section 2.2.6. Genomic DNA concentrations were normalised to 20ng/µl.

3.2.6 Nuclear receptors and cytochrome P450 genotyping

The gDNA samples were prepared for real-time qPCR as follows: 20×Primer mix (1.25µl 1.8µM final concentration), 20×Probe mix (1.25µl 0.4µM final concentration) 2×qPCR Master Mix (12.5µl) and gDNA (2µl) (Table 3.2). Reaction mixtures were then made up to 25µl final volume using DNase free water.

Thermal cycling conditions consisted of 15 minutes at 95℃ followed by 50 cycles of 15 seconds at 95℃ and 1 minute at 60℃. Samples were then held at 4℃. Quantification of PCR products occurred in real-time and was analysed using the Chromo4 Real-Time Detection System (MJ Research, INC. Waltham, Massachusetts, USA).

Table 3.2 TaqMan assay IDs and primer-probe sequences for the assays of genotype

SNPs	**TaqMan Assay ID**
PXR44477 T>C	C_26079845_10
PXR63396 C>T	C_9152783_20
PXR69789 A>G	C_1834250_10
CAR540 C>T	C_25746794_20
	Primer-probe sequences
CYP2B6 516 G>T	Forward: CTTGACCTGCTGCTTCTTCCTA
	Reverse: AGACGATGGAGCAGATGATGTTG
	FAM: TTCCATTCCATTACCG
	VIC: TTCCAGTCCATTACCG
CYP2B6 1459 C>T	Forward: CCACACTGGTGACCTTCTGT
	Reverse: CTTCCCTCAGCCCCTTCAG
	FAM: AGGAAGCAGATCTG
	VIC: AGGAAGCGGATCTG
CYP3A4 392 A>G	Forward: AGTGGAGCCATTGGCATAAAATCT
	Reverse: TGGAATGAGGACAGCCATAGAGA
	FAM: AAGGGCAAGAGAGAG
	VIC: CAAGGGCAGGAGAGAG

3.2.7 Statistical analysis

The gene expression of cytochrome P450 and nuclear receptors were log10 transformed and given as median (range) unless stated otherwise. Distribution of the gene expression of cytochrome P450 was determined via Shapiro-Wilk test. The effects on the gene expression of cytochrome P450 were assessed by Mann-Whitney test (for genotype groups) or by simple linear regression (continuous data such as the gene expression of nuclear receptors) using Pearson's rank correlation coefficient. Rho is a non-parametric correlation coefficient. A p-value < 0.05 was considered to identify groups that were significantly different from each other and a p-value < 0.1 was used as a cut off for trend. The genotypes with rare double variants (defined by < 3 in the total sample population) were treated as one genotype with the heterozygous variants in these analyses. These statistical analyses were conducted using Stats Direct (Stats Direct Ltd., Cheshire, UK)

Multivariate analysis was conducted to construct a predictive model using patient demographic, nuclear receptor gene expression and genotypes as independent variables and the gene expression of cytochrome P450 as the dependent variable. Dichotomous variables, including gender, were coded as 0 for female and 1 for male, while *PXR*44477, *PXR63396*, *PXR*69789, *CAR C>T* (rs2307424), *CYP2B6 G516T*, *CYP2B6 C1459T* and *CYP3A4 A392G* genotypes were coded as 0, 1 and 2 for homozygous wild type, heterozygous variants and homozygous variants, respectively. Ethnicity was also coded as 0, 1 and 2 for White British, Black African and others

respectively, while age was included as a continuous variable. Univariate analysis was used to select independent variables with p-values < 0.2 to be included in the multivariate analysis. These statistical analysis were conducted using SPSS 20 (SPSS Inc., Chicago, IL, USA)

3.3 RESULTS

3.3.1 Patient demographics

84 patients were recruited from the Royal Liverpool University Hospital. Patient characteristics, including age, gender and ethnicity, were obtained from medical records. The group comprised with 56% males and 43% females (one person no record). The average age of patients was 60.3 years old with a range from 17 to 88 years old. The ethnicity included 89.3% White British, 3.6% African and 7.2% others (Table 3.3).

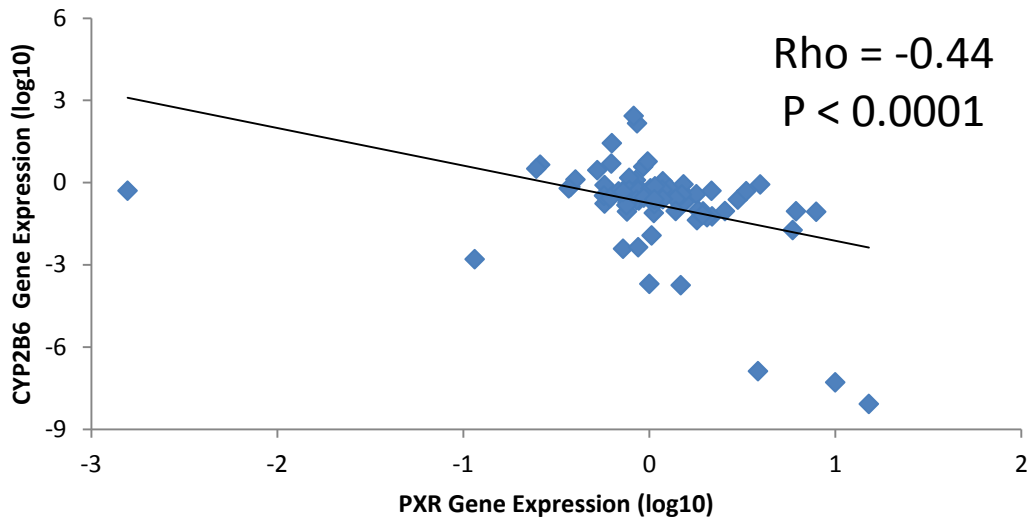
Table 3.3 demographics of patient

Demographics		
Age (y)	Average	60.3 ± 17.6
	Range	17 to 88
Gender (n, %)	Male	47，56
	Female	36，43
	No record	1, 1
Ethnicity (n, %)	White British	75，89.3
	Black African	3，3.6
	others	6，7.2

3.3.2 Correlation of nuclear receptor and cytochrome P450 expression

The correlation of gene expression in intestine between nuclear receptors (PXR and CAR) and cytochrome P450 (CYP2B6 and CYP3A4) were analysed. Negative correlations of PXR with CYP2B6 (Rho = -0.44, $P < 0.0001$) and PXR with CYP3A4 (Rho = -0.61, $P < 0.0001$) emerged (Figure 3.1). A relationship between CAR and CYP3A4 (Rho = -0.51, $P < 0.0001$) was also evident, while no other significant correlations were observed between CAR and CYP2B6 (Figure 3.2).

(a)



(b)

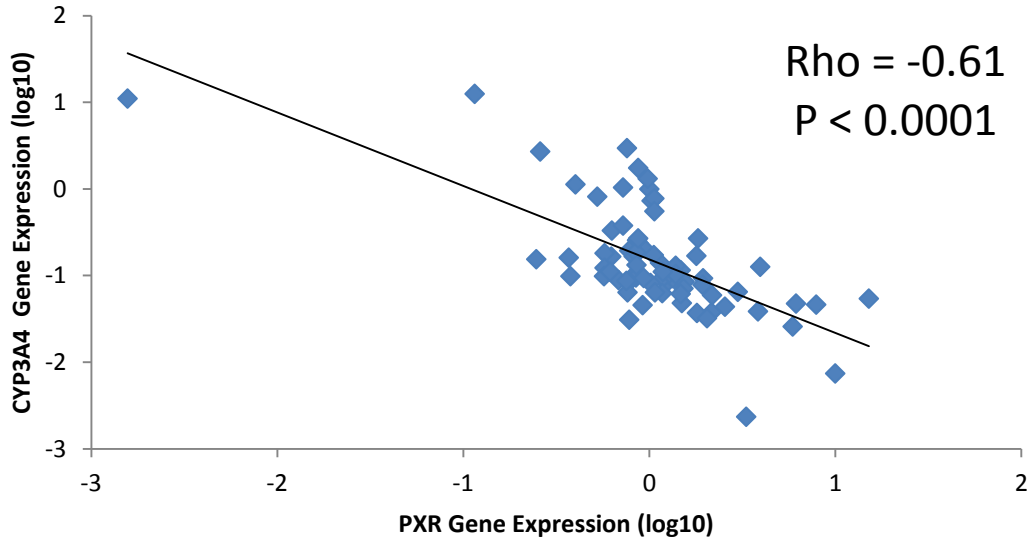
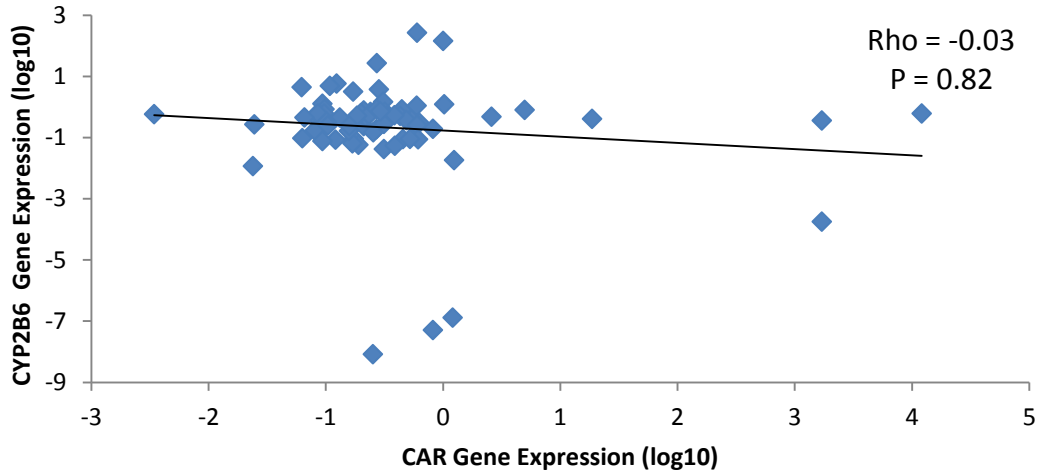


Figure 3.1 Correlation of gene expression between PXR and cytochrome P450 in intestine. Correlation between the gene expression of (a) PXR & CYP2B6 and (b) PXR & CYP3A4

(a)



(b)

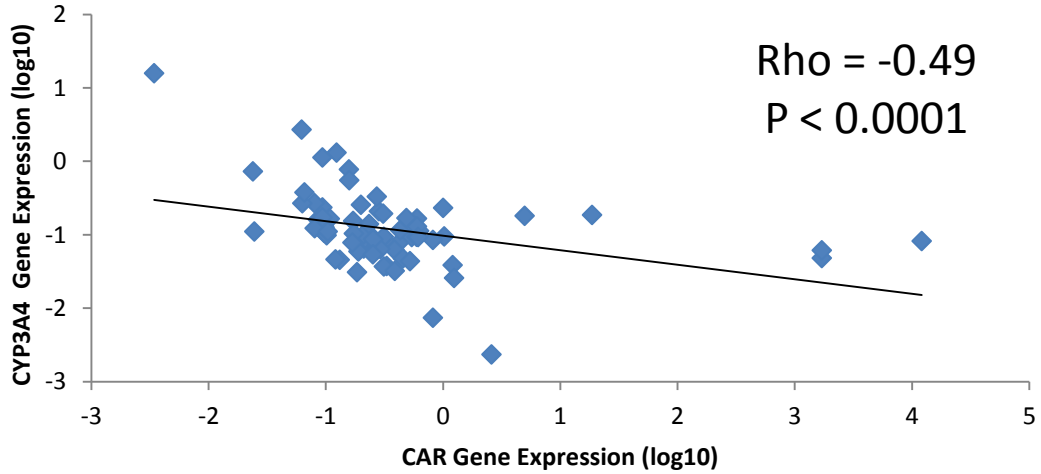


Figure 3.2 Correlation of gene expression between CAR and cytochrome P450 in intestine. Correlation between the gene expression of (a) CAR & CYP2B6 and (b) CAR & CYP3A4

3.3.3 Allele frequencies for PXR, CAR and CYPs polymorphisms

Three PXR polymorphisms (*PXR44477*, *PXR63396* and *PXR69789*) and one *CAR*540 polymorphism were investigated in the 84 patients. The results were shown in section 2.3.4. Two CYP2B6 polymorphisms (*CYP2B6 G516T* and *C1459T*) were investigated in the 84 patients. Allele frequencies for the *CYP2B6 G516T* polymorphism in intestine were 0.74 for the G allele and 0.26 for the T allele (Table 3.4). The *CYP2B6 C1459T* polymorphism allele frequencies were 0.83 for the C allele and 0.17 for the T allele (Table 3.4). The *CYP3A4 A392G* polymorphism was found at a frequency of 0.95 for the A allele and 0.05 for the G allele (Table 3.4). The observed genotype frequency was in Hardy-Weinberg equilibrium.

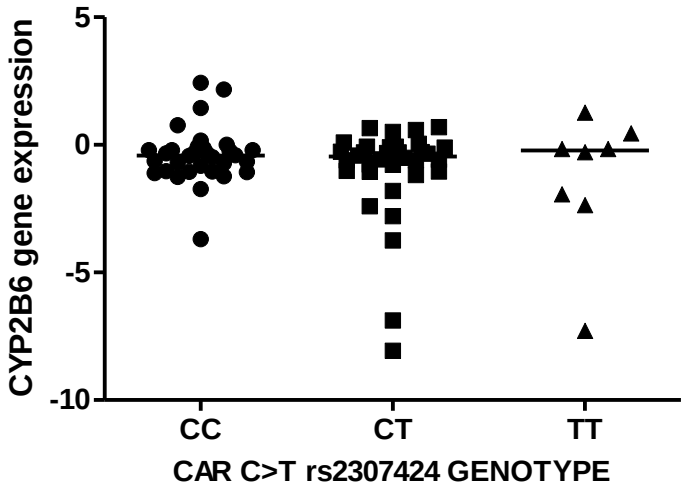
Table 3.4 Frequencies of nuclear receptor and cytochrome P450 polymorphisms

SNPs	**Genotype Frequency n (%)**			**Allele Frequency %**	
CYP2B6 516	**TT**	**GT**	**GG**	**T**	**G**
G>T	0 (0)	40 (52.6)	36 (47.4)	26.3	73.7
CYP2B6 1459	**TT**	**CT**	**CC**	**T**	**C**
C>T	0 (0)	27 (34.6)	51 (65.4)	17.3	82.7
CYP3A4 392	**AA**	**AG**	**GG**	**A**	**G**
A>G	72 (92.3)	5 (6.4)	1 (1.3)	95.5	4.5

3.3.4 Nuclear receptors polymorphisms and CYPs expression

There were no significant associations between CAR & PXR (*PXR44477*, *PXR69789* and *PXR63396*) polymorphism and gene expression of CYP2B6 and CYP3A4 in intestine (Figure 3.3 and 3.4). Similarly, no significant associations were found between cytochrome P450 polymorphisms *(CYP2B6 G516T*, *CYP2B6 C1459T* and *CYP3A4 A392G*) and gene expression of CYP2B6 or CYP3A4 in intestine (Figure 3.5 and 3.6).

(a)



(b)

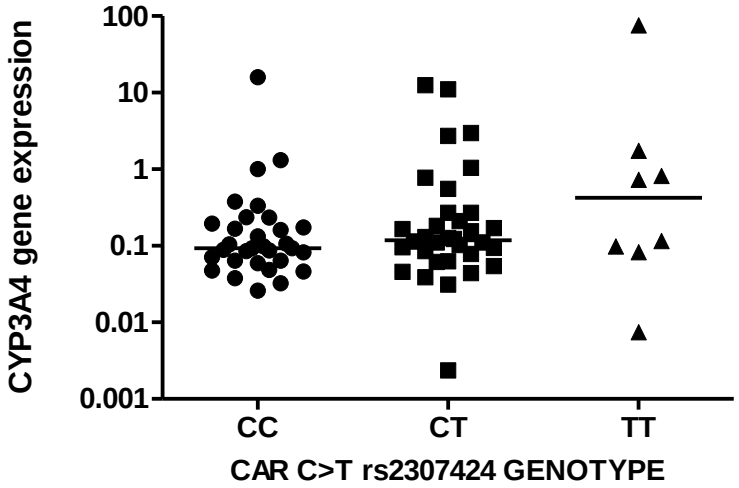
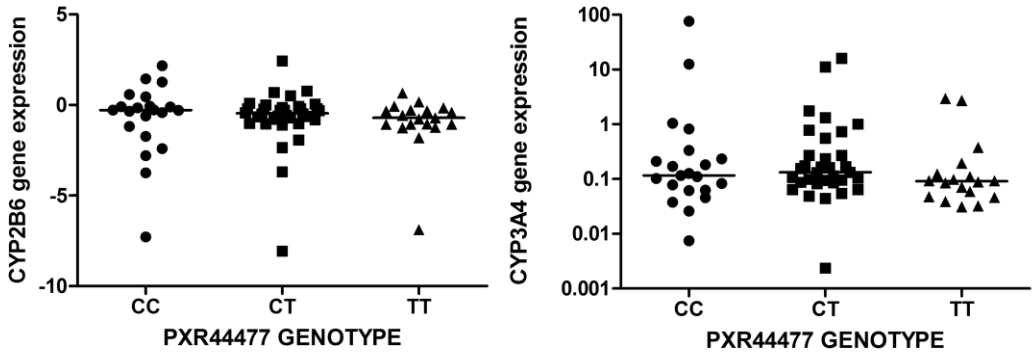
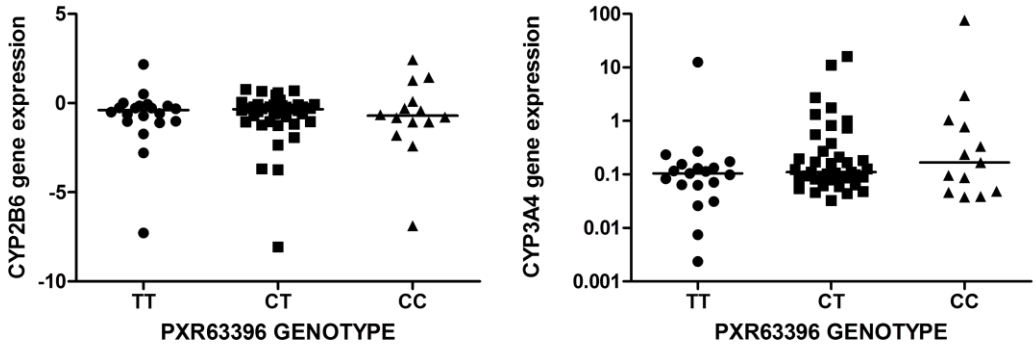


Figure 3.3 Expression of cytochrome P450 in intestine according to CAR genotypes. Expression of CYP2B6 (a) and CYP3A4 (b) according to CAR genotype and the lines indicate the median values.

(a)



(b)



(c)

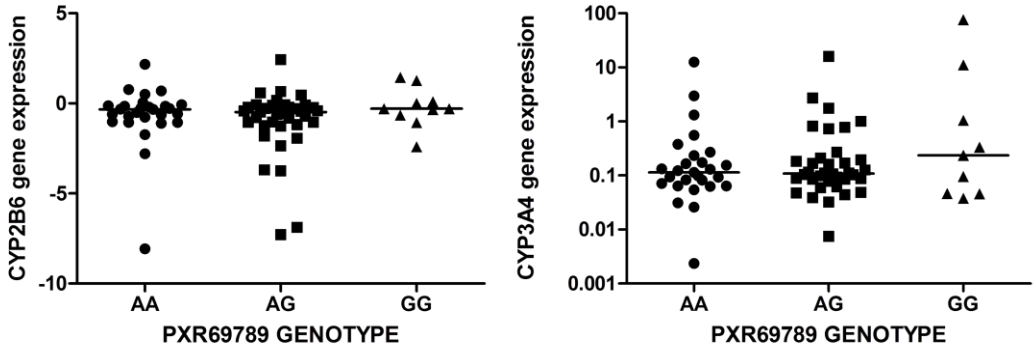
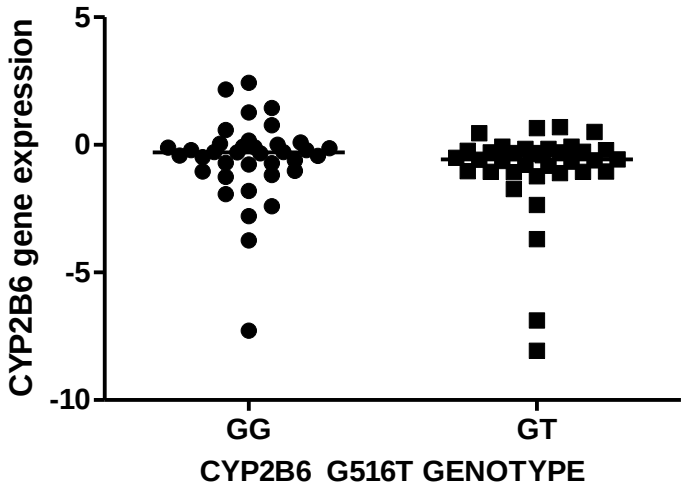


Figure 3.4 Expression of cytochrome P450 in intestines according to PXR genotypes. Expression of CYP2B6 and CYP3A4 in intestines according to *PXR44477* genotype (a), *PXR63396* genotype (b) and *PXR69789* genotype (c) and **t**he lines indicate the median values.

(a)



(b)

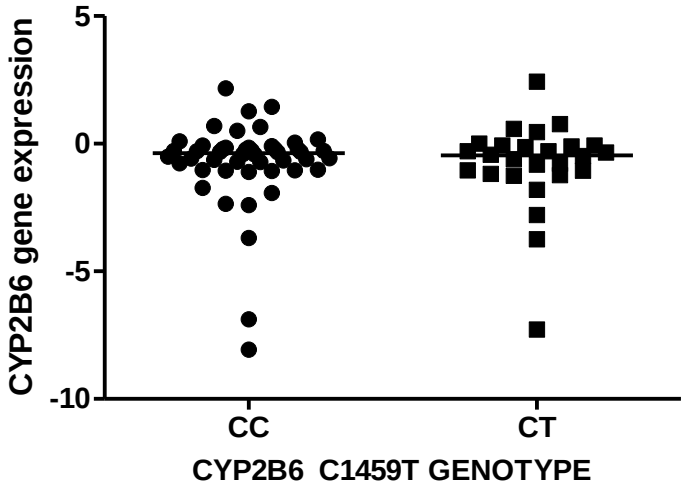


Figure 3.5 Expression of CYP2B6 in intestines according to CYP2B6 genotypes. Expression of CYP2B6 according to *CYP2B6 G516T* genotype (a), and *CYP2B6 C1459T* genotype (b) and the lines indicate the median values.

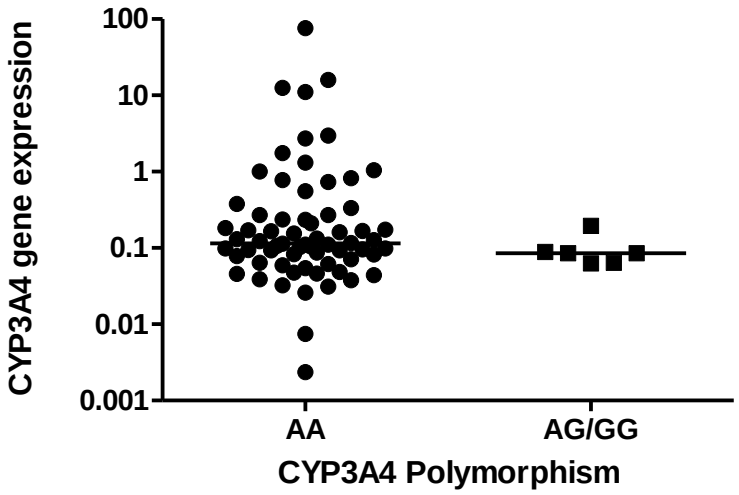


Figure 3.6 Expression of CYP3A4 in intestines according to *CYP3A4 A392G* genotypes. The lines indicate the median values.

3.3.5 Multivariate analysis of cytochrome P450 gene expression

3.3.5.1 Patient characteristics and nuclear receptor expression

Using multiple linear regression, associations of cytochrome P450 (CYP2B6 and CYP3A4) gene expression with patient characteristics (age, gender and ethnicity), nuclear receptor (PXR and CAR) gene expression were analysed and are summarised in Table 3.5. In univariate and multivariate analysis, CYP2B6 and CYP3A4 gene expression were not associated with the age, gender, ethnicity, PXR expression or CAR gene expression (Table 3.5).

Table 3.5 Univariate and multivariate linear regression analysis of cytochrome P450 gene expression with patient characteristics and nuclear receptor gene expressions

Covariate	CYP2B6 gene expression Univariate		CYP2B6 gene expression Multivariate	
	β	**P**	**β**	**P**
Age	-0.3 (-0.7 to 0.2)	0.30	-	-
Gender	2 (-15 to 18)	0.85	-	-
Ethnicity	-4 (-22 to 15)	0.70	-	-
CAR expression	-0.001 (-0.007 to 0.005)	0.82	-	-
PXR expression	-1 (-5 to 3)	0.55	-	-
Covariate	**CYP3A4 gene expression Univariate**		**CYP3A4 gene expression Multivariate**	
	β	**P**	**β**	**P**
Age	0.006 (-0.1 to 0.1)	0.93	-	-
Gender	2 (-3 to 6)	0.46	-	-
Ethnicity	-0.7 (-5 to 4)	0.77	-	-
CAR expression	-0.0002 (-0.002 to 0.001)	0.81	-	-
PXR expression	-0.5 (-1 to 0.4)	0.27	-	-

3.3.5.2 Patient characteristics and nuclear receptor polymorphisms

Associations between the gene expression of cytochrome P450 (CYP2B6 and CYP3A4) with patient characteristics and nuclear receptor polymorphisms in intestine were analysed by using multiple linear regression, which are summarised in Table 3.6. In univariate analysis, CYP3A4 gene expression was significantly associated with *CAR540* polymorphism and had a trend towards correlation with *PXR69789* polymorphism (Table 3.5). However, there was only a trend towards association between CYP3A4 gene expression and CAR polymorphism in multivariate analysis (Table 3.5).

Table 3.6 Univariate and multivariate linear regression analysis of cytochrome P450 gene expression with patient characteristics and nuclear receptor polymorphisms

Covariate	CYP2B6 gene expression Univariate		CYP2B6 gene expression Multivariate	
	β	**P**	**β**	**P**
Age	-0.3 (-0.7 to 0.2)	0.30	-	-
Gender	2 (-15 to 18)	0.85	-	-
Ethnicity	-4 (-22 to 15)	0.70	-	-
CAR540	-8 (-21 to 4)	0.19	-9 (-21 to 4)	0.18
PXR44477	5 (-7 to 16)	0.42	-	-
PXR63396	6 (-7 to 18)	0.36	-	-
PXR69789	-0.03 (-12 to 12)	0.99	-	-
CYP2B6G516T	-13 (-29 to 4)	0.12	-13 (-29 to 3)	0.11
CYP2B6C1459T	7 (-11 to 24)	0.45	-	-
Covariate	**CYP3A4 gene expression Univariate**		**CYP3A4 gene expression Multivariate**	
	β	**P**	**β**	**P**
Age	0.006 (-0.1 to 0.1)	0.93	-	-
Gender	2 (-3 to 6)	0.46	-	-
Ethnicity	-0.7 (-5 to 4)	0.77	-	-
CAR540	3 (0.1 to 7)	**0.043**	3 (-0.09 to 7)	0.057
PXR44477	2 (-0.8 to 5)	0.15	1 (-2 to 4)	0.52
PXR63396	2 (-0.9 to 5)	0.16	2 (-2 to 5)	0.40
PXR69789	3 (-0.08 to 6)	0.056	2 (-2 to 6)	0.32
CYP3A4A392G	-1 (-8 to 5)	0.65	-	-

3.4 DISCUSSION

Nuclear receptors are transcription factors that regulate the expression of cytochrome P450 enzymes. In this study, a negative significant correlation was found between PXR and CYP2B6 gene expression in intestine. While a positive and statistically significant correlation was been reported between CYP2B6 with CAR and CYP2B6 with PXR gene expression in liver (Chang *et al.*, 2003). A previous study also showed the nuclear receptor (PXR and CAR) gene expression is significantly correlated with CYP3A expression in human fetal and pediatric liver (Vyhlidal *et al.*, 2006). A positive correlation between hepatic PXR and CYP3A4 gene expression has been reported (Lamba *et al.*, 2010). However, our study obtained a negative significant correlation between CYP3A4 gene expression and the gene expression of PXR and CAR in intestine. The reasons for this difference between liver and intestine require for the study but may involve differences between expressions of other transcription factors such as Vitamin D between these tissues. In literature, PXR dominantly controls CYP3A4 inducibility in the liver, whereas Vitamin D receptor transactivates CYP3A4 in the intestine by secondary bile acids (Pavek *et al.*, 2010).

This study also investigated an association between nuclear receptor polymorphisms (*PXR44477*, *PXR69789* and *PXR63396* and *CAR C>T* [rs2307424]) and the gene expression of cytochrome P450 enzymes (CYP2B6 and CYP3A4) in intestine but no statistically significant associations emerged. In the literature, *PXR63396*

polymorphism has been reported to be associated with CYP3A4 gene expression in liver which is known to be involved in atazanavir clearance (Schipani *et al.*, 2010). Another PXR polymorphism (*PXR A566C*) was significantly associated with CYP3A4 RNA expression in colon tumour (King *et al.*, 2007).

The association between CYP2B6 and CYP3A4 polymorphisms (*CYP2B6 G516T*, *CYP2B6 C1459T* and *CYP3A4 A392G*) and the gene expression of cytochrome P450 enzymes (CYP2B6 and CYP3A4) in intestine was also investigated. No association emerged which is different from previous studies. For example, *CYP2B6 C1459T* significantly reduced CYP2B6 protein expression in liver (Lang *et al.*, 2001), while *CYP3A4 A392G* has been shown to be in association with altered CYP3A4 gene expression, also in liver (Svard *et al.*, 2010). *CYP2B6 G516T* has been correlated to changes in plasma drug concentrations of efavirenz and nevirapine in patients (Saitoh *et al.*, 2008; Svard *et al.*, 2010). *CYP2B6 C1459T* was also associated with the lowest level of CYP2B6 activity in the livers of females (Lamba *et al.*, 2003).

In multivariate linear regression analysis, *CAR C>T* (rs2307424) polymorphism independent significantly influenced the CYP3A4 gene expression, while CYP3A4 gene expression also had a trend to be associated with *PXR69789* polymorphism. The patient characteristics including age, gender and ethnicity had no influence on CYP2B6 or CYP3A4 gene expression. Similarly, no association between nuclear receptor gene expression and CYP2B6 or CYP3A4 gene expression were observed in

this analysis.

In summary, the expression of cytochrome P450 enzymes was negatively affected by nuclear receptor gene expression in intestine. CYP3A4 expression was significantly associated with *CAR C>T* (rs2307424) polymorphism in univariate analysis but there was only a trend towards association in multivariate analysis. Meanwhile, CYP3A4 expression also had a trend to be associated with *PXR69789* polymorphism in univariate analysis. No genetic associations with CYP2B6 and CYP3A4 expression were observed. Clearly, these data indicate significant differences in regulation of CYP2B6 and CYP3A4 in liver/intestinal tumours compared to intestine that are now worthy of further study.

CHAPTER 4

Impact of drug concentrations on the viral load in patients receiving highly active antiretroviral therapy

Contents

4.1 INTRODUCTION

Efavirenz (EFV) is a non-nucleoside reverse transcription inhibitor (NNRTI) and is used as part of highly active antiretroviral therapy (HAART) for the treatment of HIV-1. It is used in combination with lamivudine/zidovudine (Combivir) or tenofovir/emtricitabine (Truvada) as the preferred NNRTI-based regimens in adults and adolescents. NNRTIs inhibit the reverse transcriptase enzyme which is an essential viral enzyme used to transcribe viral RNA into DNA. NNRTIs do not bind to the active site of the polymerase but in a less conserved pocket near the active site. Their binding results in a conformational change in the reverse transcriptase that distorts the positioning of the residues that bind DNA, inhibiting polymerization (De Clercq, 1998).

EFV is metabolized in the liver, and is a substrate and inducer of CYP2B6 and possibly CYP3A4 (Asimus *et al.*, 2009; Berruet *et al.*, 2005). Large coefficients of variation in plasma levels are observed. For example, EFV intrapatient concentration variation is 30% and interpatient variation is 118% (Marzolini *et al.*, 2001). Plasma concentrations of the NNRTIs have been shown to correlate with virological response (Fabbiani *et al.*, 2009) and the relationship between EFV pharmacokinetics and pharmacodynamics has been investigated in numerous clinical studies. In 2001, Marzolini *et al* described an association between EFV plasma exposure with the probability of viral suppression and side effects in 130 patients (Marzolini *et al.*,

2001). These data have been further expanded in a cohort of 235 patients and a clear association between EFV plasma exposure and efficacy was observed (Csajka *et al.*, 2003). Patients with EFV concentrations below a cut-off of 1000 ng/ml had a much higher probability of therapeutic failure compared to patients with higher EFV concentrations. Similar observations were found in other clinical studies on 68 patients (Stahle *et al.*, 2004) and 300 patients (Leth *et al.*, 2006). However, in a more recent study of 71 patients (3 of which had therapeutic failure) this was not replicated (Josephson *et al.*, 2010). Although we do not have a complete understanding of the link between EFV exposure and CNS side effects, several studies have identified an association between plasma concentrations and CNS toxicity (Clifford *et al.*, 2005; Csajka *et al.*, 2003; Gallego *et al.*, 2004; Gutierrez *et al.*, 2005; Johnson *et al.*, 2011; Marzolini *et al.*, 2001; Nanzigu *et al.*, 2011; Nunez *et al.*, 2001; van Luin *et al.*, 2009; Wintergerst *et al.*, 2008).

Therapeutic drug monitoring (TDM) is the practice of directly measuring and managing plasma drug levels. It represents one approach to addressing uncertain concentrations resulting from standard dosing regimens. For antiretroviral therapy in HIV disease, it is used particularly for PIs and NNRTIs (Wertheimer *et al.*, 2006). The therapeutic drug monitoring of EFV may be used to improve the potency and the durability of the drug regimen, to identify patients with sub-optimal adherence and to minimise drug toxicity (Katsounas *et al.*, 2007; Maitland *et al.*, 2005).

The decision on when to start treatment should take into account CD4 count, HIV viral load, co-infections, disease status, treatment history, resistance profiles and patient preference. Viral load is the measure of the severity of a viral infection, and can be calculated by quantifying the amount of virus in an involved body fluid. Tracking viral load is used to monitor therapy during chronic viral infections, such as HIV-1, cytomegalovirus, hepatitis B virus, and hepatitis C virus (Nijman *et al.*, 2012; Pardo-Yules *et al.*, 2012; Parisi *et al.*, 2012; Tseng *et al.*, 2012). An HIV viral load greater than 100,000 copies/ml of blood within six months of seroconversion indicates a greater likelihood of developing AIDS within five years (Clinlab-Navigator, 2012). Conversely, a viral load less than 10,000 copies/ml in the early stages indicates a better prognosis with decreased risk of developing AIDS (Engels *et al.*, 1999).

Successful combination therapy should give a fall in viral load of 1.5 to 2 logs (30-100 folds) within six weeks, with the viral load falling below the limit of detection within four to six months (AIDSinfo, 2012). The limit of quantification for viral load was initially 400 copies/ml with first-generation tests, and later 50 copies/ml with second-generation tests following the introduction of viral load monitoring in clinical practice (Doyle *et al.*, 2012). Extensive evidence from clinical trials and observational studies has shown that maintaining a viral load lower than 50 copies/ml predicts long-term virologic suppression and sustained immunologic and clinical benefit (Geretti *et al.*, 2008; Margot *et al.*, 2009; Mocroft *et al.*, 2007).

In this study, we investigated the virologic outcomes of treated patients showing a viral load lower than 50 copies/ml at an arbitrarily selected time point during HAART (time zero, $_{T0}$VL) according to whether the actual, unreported $_{T0}$VL result showed a level between RNA detected (RNA+) or RNA not detected (RNA-). We also investigated the EFV concentration of $_{TO}$VL plasma samples and compared them as two groups (RNA+ and RNA-). The aim of this study was to find the influence between patients' demographics (age, gender and ethnicity) and EFV plasma concentrations with the impact of EFV plasma concentration on the $_{T0}$VL in patients receiving HAART.

4.2 METHODS

4.2.1 Materials and Instruments

Efavirenz (EFV) was donated by DuPont Pharmaceuticals Company (Wilmington, USA). Blank plasma was obtain form the National Blood Service. Acetonitrile (ACN), Methanol (MeOH), Ethyl acetate [Cat no 152516Q] and n-hexane were purchased from VWR International Ltd. (Leicestershire, UK). Potassium carbonate (K_2CO_3) and ammonium acetate were purchased from Sigma Aldrich (Poole, UK). Internal standard Ro 31-9564 were purchased from Roche Discovery (Welwyn, UK). Deionised water was obtained from the reservoir of the Option 4 water purifier (Elga, High Wycombe, Bucks, and UK). The HPLC consisted of a Dionex (Dionex Softron GmbH, Germany) HPLC system with a P 680 pump, ASI-100 automated sample injector and UVD170U detector. Reversed-phase-liquid chromatography was carried out using a Hypurity C_{18} analytical column, 5μm 150 × 4.6mm from Thermo Electron Corporation (Runcorn, UK) with a pre-column guard (Si 60 × 5 mm; Merck, New Jersey, USA).

4.2.2 Patients Data

The study population comprised 187 patients from the Royal Free Hospital (Table 4.3). Patient characteristics, including age, gender and ethnicity, were obtained from medical records. The group was comprised of 144 male and 43 female patients. The average age of patients was 44.2 years old with a range of 25.4 to 67 years old. The ethnicity of these patients included White, Black and others. The patients at an arbitrary time point during HAART (T0) showed one of the following VL results: RNA+ (n = 91) or RNA- (n = 96).

4.2.3 Preparation of Reagents, Standards and Quality Controls

- **Rinsing and Extraction solvent**

Rinsing solvent was composed of deionised water and ACN (50:50 v/v) and extraction solvent was composed of ethyl acetate and n-hexane (50:50 v/v).

- **Stock Potassium Carbonate Buffer and Ammonium Acetate Buffer**

Stock potassium carbonate buffer (1M) was prepared with 69.1g potassium carbonate in 500ml deionised water and ammonium acetate buffer (1M) was prepared with 38.54g ammonium acetate in 500ml deionised water. Both of them were stored at room temperature.

- **Mobile Phase**

4ml of 1M ammonium acetate buffer was diluted with 400ml deionised water. 95ml methanol was added and then made up to 1000ml with ACN. The mobile phase for the analysis was composed of ammonium acetate, ACN and MeOH (400:505:95 v/v) and was prepared fresh for each assay.

- **EFV Stock Standard**

1mg/ml solution of EFV was prepared by weighing out approximately 5mg of drug into a 10ml glass tube with the equivalent volume of methanol added. Then 0.8ml of this EFV solution was moved into a 100ml flask with blank plasma up to the mark to make the 8μg/ml EFV plasma stock solution. 900μl of plasma was moved into the 0.2ml screw cap micro tubes and stored at -20 ℃.

- **Working Internal Standard**

10mg internal standard (Ro 31-9564) was dissolved in 10ml methanol to prepare the stock internal standard (1mg/ml) which was stored at 4 ℃ in a 10ml capped tube. 2.5 ml of stock internal standard and 7.5ml methanol were used to prepare fresh working internal standard (250μg/ml) for each assay.

- **Quality Controls (QCs)**

1mg/ml solution of EFV was prepared by weighing out approximately 5mg of drug into 10ml glass tube with the appropriate volume of methanol added. Three 50ml flasks were labelled with low, medium and high. The correct volume of each drug was moved into each flask (Table 4.1). Then bank plasma was added to each flask up to the mark. Finally, 500μl of plasma was moved to screw cap micro tubes and stored at -20 ℃.

Table 4.1 Quality controls: concentrations and volumes of EFV

Flasks	EFV	
	Concentration (ng/ml)	**Volume (µl) of 1mg/ml**
Low	*800*	40
Medium	*4000*	200
High	*12000*	600

4.2.4 Quantification of efavirenz

Validation of the optimized HPLC method was done with following parameters: specificity (UV diode array detector), linearity (three preparations at each of the 80, 100 and 120% levels), Range (80-120%), Accuracy (three preparations at each level), Precision (repeatability studies), detection limit, quantitation limit and stability in solution. In this study, EFV plasma levels were determined by the use of reverse phase HPLC with UV detection. EFV was extracted from patient plasma samples with ethyl acetate and n-hexane. The extract was then dried and reconstituted with the mobile phase. Separation was achieved by the relative non polar interactions of the components of interest (EFV) with the hydrophobic stationary phase and mobile phase. Compounds were separated into individual peaks with more hydrophobic analytes being retained longer on the column. Ultra-violet detection was used to measure concentration of EFV. Compounds differentially absorb light at a fixed wavelength. Absorbance is proportional to the concentration of the analyte. EFV concentrations were determined by comparison to a calibrator of known concentration. An internal standard solution was included in the sample extraction procedure to correct for any variation in extraction efficiency.

The stock EFV, QCs and patient samples were centrifuged at 2000 rpm for 4min. 50µl stock EFV was diluted by 450µl blank plasma to prepare a 1:10 diluted stock EFV. The correct volume of blank plasma, stock EFV and diluted stock EFV were moved into each tube by using the formula in Table 4.2. 200µl QCs (low, medium and high)

and patient samples were moved into 7ml glass tube. Internal standard (20µl, 250µg/ml) was added to plasma samples (200µl) and standards (range 100–8000ng/ml; 200µl). Potassium carbonate (100µl, 1M) and ethyl acetate/n-hexane (50:50 v/v; 3ml) were added to each tube and the samples tumbled for 30 min using a rotary mixer. Following centrifugation (4000×g; 5min), the aqueous layer was frozen using a cryogenic bath, the organic layer transferred into clean glass tubes and evaporated to dryness. Dried extracts were then reconstituted in mobile phase (150µl), vortexed thoroughly and transferred into autosampler vials ready for injection (50µl) in the HPLC system. Recovery of efavirenz, after extraction from plasma, using this method is 98%. Efavirenz and IS were resolved on a Hypurity C18 column with a pre-column guard with an HPLC system using the mobile phase (1mM ammonium acetate buffer/ACN/methanol; 400:505:95; by vol.) at a flow rate of 1.2 ml/min. The ultraviolet detector was set to monitor the 250nm wavelength. Efavirenz and IS eluted at 5.3 and 13.5 min, respectively. The peak areas for efavirenz and internal standard were quantified using the Chromeleon data acquisition system (Dionex Corporation, California, USA). The lower limit of quantification of efavirenz in plasma was taken as the lowest point on the standard curves (110ng/ml for the standard curves).

Table 4.2 Calibration standards: concentrations and volumes of EFV

Tube number	Concentration (ng/ml)	Diluted stock EFV (µl)	Blank plasma (µl)
1,2	0	0	200
3,4	100	25	175
5,6	200	50	150
7,8	500	125	75
Tube number	**Concentration (ng/ml)**	**Stock EFV (µl)**	**Blank plasma (µl)**
9,10	1000	25	175
11,12	2000	50	150
13,14	4000	100	100
15,16	8000	200	0

4.2.5 Statistical analysis

The plasma concentration of EFV was log10 transformed and given as median (range) unless stated otherwise. Distribution of the EFV plasma concentrations was determined via Shapiro-Wilk test. The effects of patient demographics and genotypes on the EFV plasma concentrations were assessed by Mann-Whitney test (for gender and ethnicity) or by simple linear regression (continuous data including age) using Pearson's rank correlation coefficient. The effect of EFV plasma concentration on VL was assessed by Mann-Whitney test. Rho is a non-parametric correlation coefficient. A p-value < 0.05 was considered to identify groups that were significantly different from each other and a p-value < 0.1 was used as a cut off for trend. These statistical analysis were conducted using Stats Direct (Stats Direct Ltd., Cheshire, UK)

Multivariate linear regression analysis was conducted to construct a predictive model using patient demographic and EFV plasma concentration as independent variables and $_{T0}$VL as dependent variable. Dichotomous variables were coded as 0 for female and 1 for male, while ethnicity was coded as 0, 1 and 2 for White, Black and others respectively and VL was also coded as 0, 1 and 2 for 40-49 copies/ml, RNA+ and RNA-, respectively. Age was included as a continuous variable. Univariate analysis was used to select the independent variables with p-values < 0.2 to be included in the multivariate analysis. These statistical analysis were conducted using SPSS 20 (SPSS Inc., Chicago, IL, USA)

4.3 RESULTS

4.3.1 Study population

187 patients were recruited from the Royal Free Hospital. Patient characteristics, including age, gender and ethnicity, were obtained from medical records. The group comprised of 77% males and 23% females. The average age of patients was 44.2 years old with a range of 25.4 to 67 years old. The ethnicity included 64.2% White, 29.9% Black and 5.9% others (Table 4.3). The patients at an arbitrary time point during HAART (T0) showed 48.7% RNA+ and 51.3% RNA-. The characteristics of the study population at T0 are summarised in Table 4.3.

Table 4.3 Characteristics of the population at study entry, defined as time zero (T0)

Demographics		
Age (y)	Average	44.2
	Range	25.4 to 67
Gender (n, %)	Male	144，77.0
	Female	43，23.0
Ethnicity (n, %)	White	120，64.2
	Black	56，29.9
	others	11，5.9
HIV RNA copies (n, %)	RNA+	91, 48.7
	RNA-	96, 51.3

4.3.2 Linearity, limit of quantification and limit of detection

The lower limit of quantification of EFV was 100.7ng/ml whereas the upper limit of quantification was 8137.1ng/ml. The concentration response relationship for EFV standard was found to be linear in the concentration range of 100.7ng/ml to 8137.1ng/ml (Figure 4.1). This linear relationship was demonstrated by coefficient of variation obtained from the daily standard curve used for the analysis of unknown samples.

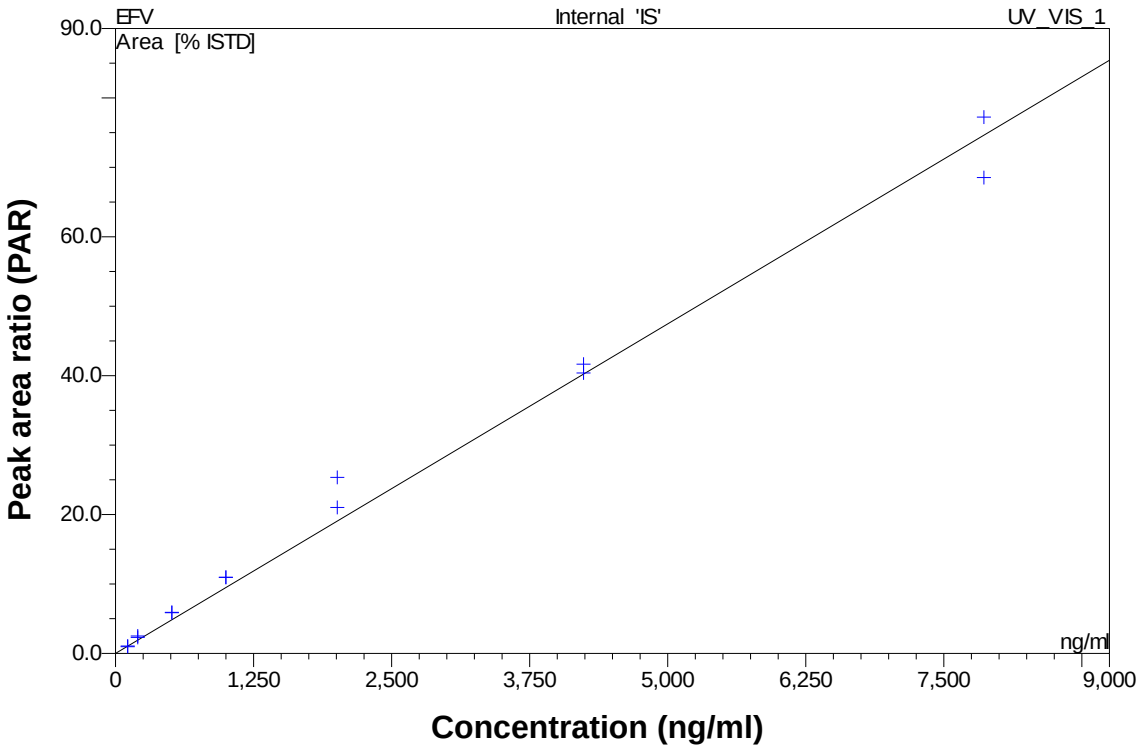


Figure 4.1 Plots of the calibration curve showing the concentration response relationship of EFV standard

4.3.3 Impact of patient demographic on EFV plasma concentration

Patient demographic including age, gender and ethnicity were investigated for their influence on EFV plasma concentration at T0. No correlation between age and EFV plasma concentration at T0 emerged (Figure 4.2). There were also no significant associations between gender and EFV plasma concentration at T0 (Figure 4.3) and no relationship was found between ethnicity and EFV plasma concentration at T0 (Figure 4.4).

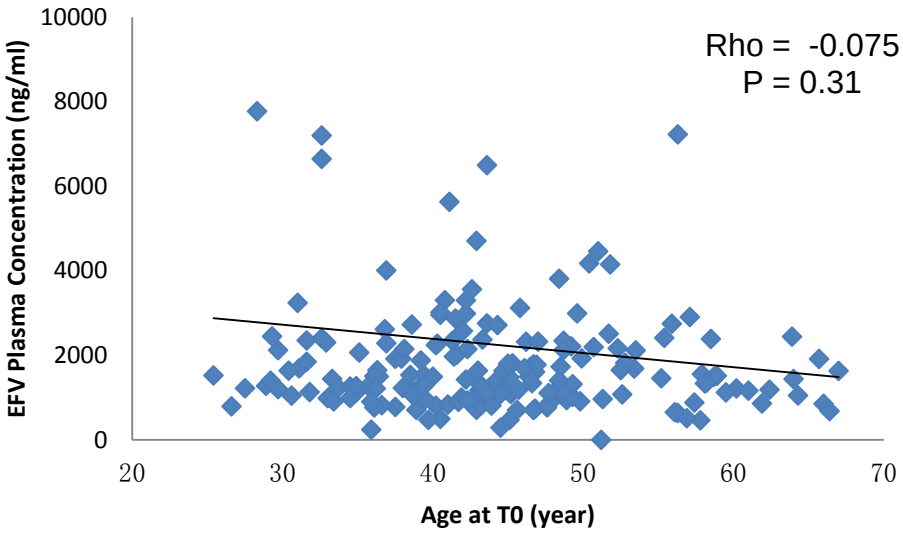


Figure 4.2 Correlation between age and EFV plasma concentration

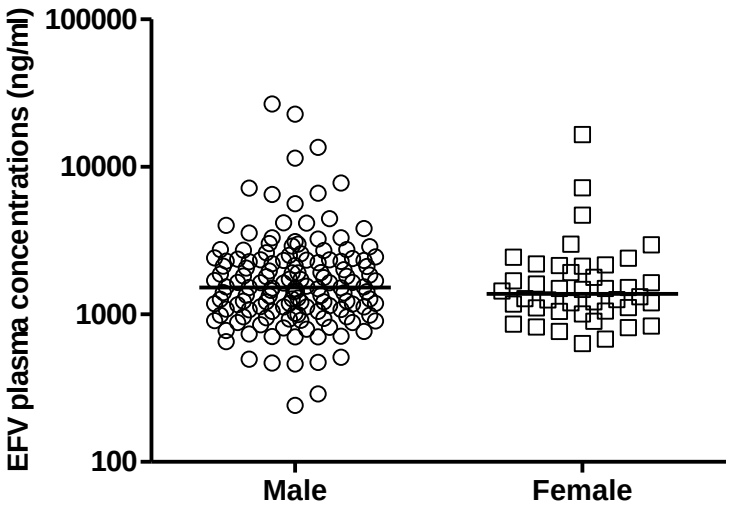


Figure 4.3 Impact of gender on EFV plasma concentration. The lines indicate the median values.

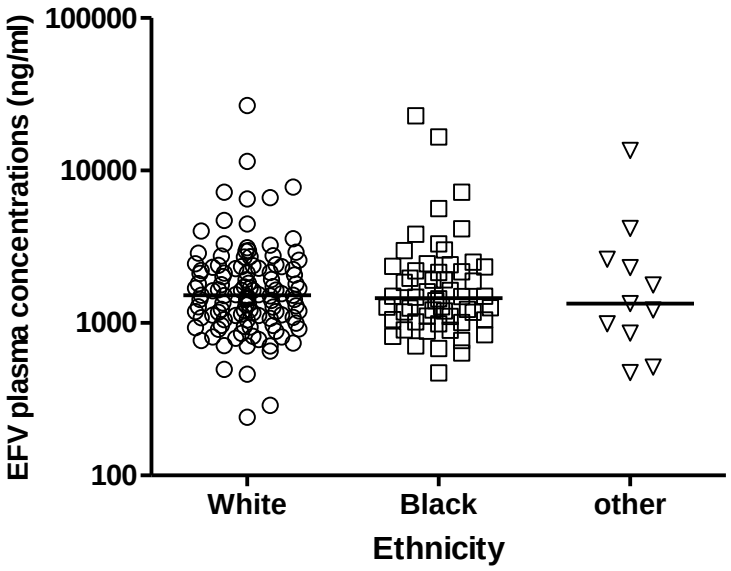


Figure 4.4 Impact of ethnicity on EFV plasma concentration. The lines indicate the median values.

4.3.4 Impact of EFV plasma concentration on VL

We investigated T0 EFV plasma levels among 187 patients receiving EFV-based HAART, comprising 91 patients with detectable HIV-1 RNA+ and 96 patients with RNA-. In these two groups, the median EFV levels at T0 were 1341.5ng/ml and 1729.7ng/ml respectively (p = 0.008). The levels were above the recommended threshold of 1000ng/ml in 63/91 (69.2%) and 81/96 (84.4%) patients respectively (p = 0.25). We also detected a significant difference in EFV plasma concentrations between the different $_{T0}$VL groups (Figure 4.5).

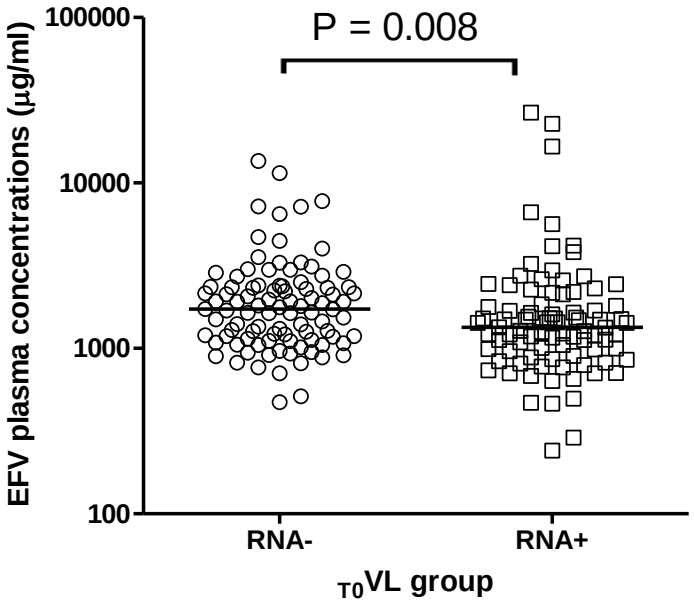


Figure 4.5 Efavirenz plasma concentrations at T0 in patients receiving Efavirenz-based HAART. The lines indicate the median values.

4.3.5 Multivariate analysis

In univariate analyses, age, gender ethnicity and EFV plasma concentration were not significantly correlated with $_{T0}$VL but gender and ethnicity had a trend towards correlation with $_{T0}$VL. In multivariate analysis, age, gender, ethnicity and EFV plasma concentration were not significantly correlated with $_{T0}$VL as shown on Table 4.4.

Table 4.4 Univariate and multivariate linear regression analyses of independent variables on the $_{T0}$VL as dependent variable

Covariate	$_{T0}$VL Univariate		$_{T0}$VL Multivariate	
	β	**P**	**β**	**P**
Age (yrs)	0.007	0.22	-	-
	(-0.004 to 0.01)			
Gender	0.24	**0.052**	0.2	0.15
	(-0.002 to 0.5)		(-0.07 to 0.4)	
Ethnicity	-0.16	**0.068**	-0.1	0.20
	(-0.3 to 0.01)		(-0.3 to 0.06)	
EFV (µg/ml)	-0.00002	0.36	-	-
	(-0.00005 to 0.00002)			

4.4 DISCUSSION

TDM is the practice of managing plasma drug concentrations and this intervention can potentially improve inadequate antiretroviral dosing in the treatment of HIV infection. Variability in plasma concentrations may result from several factors, including adherence, absorption, metabolism, clearance, and drug-drug interactions (Back *et al.*, 2002). EFV concentration ranges of 1–4mg/L are achievable thresholds, with evidence for improved viral suppression and reduced CNS toxicity (Marzolini *et al.*, 2001; Stahle *et al.*, 2004). This relationship justifies the use of EFV plasma quantification in HAART management and optimization.

In this chapter, the patients' demographics including age, gender, and ethnicity had no influence on EFV plasma concentrations. Previous studies showed the CYP2B6 516G> T polymorphism increased plasma efavirenz concentrations (Gounden *et al.*, 2010; Puthanakit *et al.*, 2009; To *et al.*, 2009). Plasma concentrations of efavirenz were also associated with body weight in HIV-positive individuals (Poeta *et al.*, 2011). Using sensitive testing methods, HIV-1 RNA can be detected in plasma in a large proportion of patients receiving HAART and showing a VL < 50 copies/ml for many years (Dornadula *et al.*, 1999; Havlir *et al.*, 2003). Viral kinetics studies have shown that plasma HIV-1 RNA levels decline to < 50 copies/ml within approximately 12 weeks of starting therapy (Di Mascio *et al.*, 2003). Once below 50 copies/ml, the levels continue to decline for several months before reaching a plateau of residual viremia at

around 3-10 copies/ml (Maldarelli *et al.*, 2007). In a previous study, EFV and indinavir decreased the HIV-1 RNA levels of patients from 10 copies/ml to levels < 2.5 copies/ml (Havlir *et al.*, 2003). Conversely, HIV-1 RNA levels of patients with a triple HAART regimen have been seen to increase gradually above 50 copies/ml when they used a monotherapy with ritonavir-boosted atazanavir (Tobin *et al.*, 2005). Furthermore, raltegravir intensification of a three-drug suppressive HAART regimen resulted in a specific and transient increase in episomal DNAs in a large percentage of HAART-suppressed subjects (Buzon *et al.*, 2010). Finally, HIV-1 genetic evolution has been observed with HIV-1 RNA levels between 6.5 and 50 copies/ml (Shiu *et al.*, 2009). For all patients with VL < 50copies/ml in this study, a significant difference of EFV plasma concentration between HIV RNA detected and undetected was observed.

However, no correlation between VL and EFV plasma concentration emerged in univariate and multivariate analysis. A trend towards correlation of gender and ethnicity with viral load was observed in univariate analysis while age, gender and ethnicity of patients were all shown not to influence on viral load of plasma in multivariate analysis. A study in univariate analysis showed a similar results with ours in gender ($p = 0.77$) and ethnicity ($p = 0.34$) but black Africans presented with a viral load lower than that of patients in other groups in multivariate analysis (Saul *et al.*, 2001). Conversely, previous study also showed Women had higher viral loads than men when CD4 T-cell counts were at most 50cells/μl (Donnelly *et al.*, 2005).

In summary, EFV plasma concentrations influenced the viral load in plasma in this cohort of patients from the UK. However, no correlation was observed between patients' demographics and EFV plasma concentration. Since intracellular concentrations of drug may be more important for viral suppression future studies should assess the relationship with concentrations at the intracellular target.

CHAPTER 5

Impact of single nucleotide polymorphisms and copy number variations on efavirenz plasma concentrations

Contents

5.1 INTRODUCTION

Combination antiretroviral therapy (cART) is increasingly used in the treatment of HIV infection. Therapeutic effect may be limited by toxicity, pill burden, need for strict adherence to treatment, emerging prevalence of resistance and the risk of developing adverse drug interactions. Effective cART comprises three or more drugs from any of the five classes: nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), Integrase inhibitors, and entry inhibitors (EI).

Efavirenz (EFV) is an essential component of the regimens for the treatment of HIV-1 infection. Recent studies provide evidence for the role of cytochrome P450 (CYP) genes, in particular CYP2B6, in relation to EFV pharmacokinetics (Heil *et al.*, 2012). CYP2B6 polymorphisms also influence the clinical progression under EFV treatment (Elens *et al.*, 2010). Previous studies showed the CYP2B6 polymorphism was significantly associated with plasma EFV concentrations (Gounden *et al.*, 2010). Particularly, *CYP2B6 516 G>T* polymorphism significantly affects the drug metabolism of EFV (Puthanakit *et al.*, 2009). Another polymorphism, *CYP2B6 983 T>C*, is very rare in Caucasians but is also strongly associated with EFV plasma concentrations (Wyen *et al.*, 2008). This polymorphism is most common in African populations with the highest frequency being reported in Ghana (Mehlotra *et al.*, 2007). The constitutive androstane receptor (CAR) is a nuclear receptor involved in

the transcriptional regulation of enzyme expression and may also contribute to the inter-individual variability in plasma EFV concentrations (Wyen *et al.*, 2011).

ABCB5 is a plasma membrane-spanning protein that in humans is encoded by the *ABCB5* gene (Frank *et al.*, 2003). It is an ABC transporter, related to P-glycoprotein and is normally expressed in various tissues and cells, and enhanced expression has been demonstrated in selected cancers (Setia *et al.*, 2012). Recent work in our laboratory has indicated that EFV is a substrate for ABCB5 (unpublished).

Copy number variations (CNVs) are a form of structural variation and alterations of the DNA of a genome that results in the cell having an abnormal number of copies of one or more sections of the DNA. CNVs are important polymorphisms that can modulate gene expression and predispose to various clinical phenotypes (Al-Sukhni *et al.*, 2012). CNVs can be caused by genomic rearrangements such as deletions, duplications, inversions and translocations. They result in the level of transcription being higher or lower than those that can be achieved by control of transcription of a single copy (Mefford *et al.*, 2011). Recently, CNVs have been associated with genetic disease such as cancer, immune disease and neurological disorders (Al-Sukhni *et al.*, 2012; Coe *et al.*, 2012; Huber *et al.*, 2011). Of particular interest, a recent study indicated that the ABCB5 gene contained functional CNVs (Glessner *et al.*, 2010).

The aim of this study was firstly to investigate the frequencies of the Single Nucleotide Polymorphisms (SNPs) of CYP2B6 (*CYP2B6 516G>T* [rs3745274] and *CYP2B6 983T>C* [rs28399499]), the *CAR540 C>T* (rs2307424) polymorphism and CNVs of ABCB5 in a cohort of Ghanaian HIV patients. The impact of these selected CYP2B6 and CAR genetic polymorphisms and CNV of ABCB5 on the plasma concentrations of EFV in a subset of patients was also assessed.

5.2 METHODS

5.2.1 Materials

EFV was donated by DuPont Pharmaceuticals Company (Wilmington, USA). Blank plasma was obtained from the National Blood Service. Acetonitrile (ACN), Methanol (MeOH), Ethyl acetate and n-hexane were purchased from VWR International Ltd. (Leicestershire, UK). Potassium carbonate (K_2CO_3) and ammonium acetate were purchased from Sigma Aldrich (Poole, UK). Internal standard Ro 31-9564 was purchased from Roche Discovery (Welwyn, UK). Deionised water was obtained from the reservoir of the Option 4 water purifier (Elga, High Wycombe, Bucks, and UK). Chloroform and nuclease free water were purchased from Sigma-Aldrich (Poole, UK). Isopropyl alcohol and ethanol were obtained from Fisher Scientific (Loughborough, UK). Whole blood DNA extraction kits were purchased from QIAGEN (West Sussex, UK). The primers and probes for real-time PCR based allelic discrimination assay and the TaqMan copy number assay were obtained from the Assays by Design Service, Applied Biosystems (Warrington, UK).

5.2.2 Instruments

The HPLC consisted of a Dionex (Dionex Softron GmbH, Germany) HPLC system with a P 680 pump, ASI-100 automated sample injector and UVD170U detector. Reversed-phase-liquid chromatography was carried out for EFV using a Hypurity C_{18} analytical column, 5µm 150 × 4.6mm from Thermo Electron Corporation (Runcorn, UK).

5.2.3 Patients Data

800 patients with confirmed HIV infection were recruited. All patients were receiving EFV-based cART. Genotype data were analysed to examine the impact of selected polymorphisms in EFV metabolising enzymes on steady state concentrations of plasma EFV. ABCB5 CNV data were analysed in a subset of patients (352) to investigate the impact of copy numbers on EFV plasma concentrations. To be included in the study, patients were aged at least 18 years and those on EFV were taking it at the fixed dose of 600mg daily plus two nucleoside reverse transcriptase inhibitors and had good pharmacy records and pill counts as surrogates for adherence.

5.2.4 Quantification of plasma EFV concentration

EFV concentrations were determined using high performance liquid chromatography (HPLC) with UV detection. The limit of detection was 110ng/ml and the upper limit was 8000ng/ml. Analyses were conducted as outlined in section 4.2.4.

5.2.5 Genotyping of CYP2B6 and CAR polymorphisms

Genomic DNA was extracted from serum using a kit from QIAGEN according to the manufacturer's instructions as outlined in section 2.2.6. Genotypes for the CYP2B6 (*CYP2B6 G516T* and *CYP2B6 T983C*) and the CAR (*CAR C>T* [rs2307424]) polymorphisms were determined by real-time PCR using standard methodology according to the manufacturer's instructions and as outlined in section 2.2.8.

Table 5.1 TaqMan assay IDs and primer-probe sequences for the assays of genotype

SNPs	**TaqMan Assay ID**
CAR540	C_25746794_20
	Primer-probe sequences
CYP2B6 G516T	Forward: CTTGACCTGCTGCTTCTTCCTA
	Reverse: AGACGATGGAGCAGATGATGTTG
	FAM: TTCCATTCCATTACCG
	VIC: TTCCAGTCCATTACCG
CYP2B6 T983C	Forward: GCCTGAAATGCCTCTTTAAAATGAGATTC
	Reverse: GCGATGTGGGCCAATCAC
	FAM: CTGTTCAATCTCCC
	VIC: CTGTTCAGTCTCCC

5.2.6 Copy number assays for ABCB5

Genomic DNA was extracted from serum using a kit from QIAGEN according to the manufacturer's instructions as outlined in section 2.2.6. Genomic DNA concentrations were normalized to 20ng/µl. Samples were prepared for real-time PCR as follows: 10µl Master mix (qPCR ROX mix), 1µl TaqMan ABCB5 CNVs assay, 1µl copy number reference assay RNase P and 3µl gDNA. Reaction mixtures were then made up to 20µl final volume using DNase free water.

Thermal cycling conditions consisted of 15 minutes at 95℃ followed by 40 cycles of 15 seconds at 95℃ and 1 minute at 60℃. Samples were then held at 4℃. Quantification of PCR products occurred in real-time and was analysed using the Chromo 4 Real-Time Detection System (MJ Research, INC. Waltham, Massachusetts, USA).

5.2.7 Statistical analysis

The plasma concentration of EFV was log10 transformed and given as median (range) unless stated otherwise. Distribution of the EFV plasma concentrations was determined via Shapiro-Wilk test. The effects of patient demographics and genotypes on the EFV plasma concentrations were assessed by Mann-Whitney test (for gender and genotype groups) or by simple linear regression (continuous data including age and body weight) using Pearson's rank correlation coefficient. Rho is a non-parametric correlation coefficient. A p-value < 0.05 was considered to identify groups that were significantly different from each other and a p-value < 0.1 was used as a cut off for trend. The genotypes with rare double variants (defined by < 3 in the total sample population) were treated as one genotype with the heterozygous variants in these analyses. These statistical analyses were conducted using Stats Direct (Stats Direct Ltd., Cheshire, UK). Multivariate analysis was conducted to construct a predictive model using patient demographic factors and genotypes as independent variables and EFV plasma concentrations as the dependent variable. Dichotomous variables, including gender, were coded as 0 for female and 1 for male, while *CYP2B6 516G>T, CYP2B6 983T>C, CAR C>T* (rs2307424) genotypes were coded as 0, 1 and 2 for homozygous wild type, heterozygous variants and homozygous variants respectively, while age and weight were included as continuous variables. Then univariate analysis was used to select the independent variables with p< 0.2 to be included in the multivariate analysis. These statistical analyses were conducted using SPSS 20 (SPSS Inc., Chicago, USA)

5.3 RESULTS

5.3.1 Patient demographics

800 patients were recruited and the patient characteristics, including age, gender weight and height, were obtained from medical records. The average age of study participants was 40.2 years old with a range from 17 to 68 years old. The ratio of female to male was 1.2:1. The average body weight of patients was 59.7kg with a range from 34kg to 102kg. The body height of patients was from 110cm to 186cm and the average was 160.9cm. 72.3% of patients were on cART while 27.7% were naïve to therapy at the time of sampling. Of those on cART, 90.1% of patients were on EFV-based therapy. Demographic data are presented in Table 5.2.

Table 5.2 demographics of patients in Ghana cohort

Demographics		
Age (y)	Average	40.2
	Range	17 to 68
Gender (n, %)	Male	363，45.4
	Female	431，53.8
Body weight (cm)	Average	59.7
	Range	34 to 102
Body height (kg)	Average	160.9
	Range	110 to 186
cART (n, %)	Efavirenz-based	521, 90.1
	Nevirapine-based	56, 9.7
	Nelfinavir-based	1, 0.2

5.3.2 Frequencies of SNPs in EFV metabolising enzymes

Of the 800 samples from which genomic DNA were extracted, genotyping was successful for 88.1% of *CYP2B6 G516T*, 87.6% of *CYP2B6 T983C* and 86.9% of *CAR540* polymorphisms respectively. Genotype frequency and allele frequency of these polymorphisms analysed in this study are shown in table 5.3. Overall, the genotype frequencies of *CYP2B6 G516T* were 29.8% of GG, 44.3% of GT and 25.9% of TT; *CYP2B6 T983C* were 90.5% of TT, 9.4% of CT and 0.1% of CC; and *CAR C>T* (rs2307424) were 86.5% of CC, 13% of CT and 0.5% of TT (Table 5.3). Allele frequencies for *CYP2B6 G516T* were allele 0.52 for the G and 0.48 for the T allele, *CYP2B6 T983C* were 0.95 for the T allele and 0.05 for the C allele and *CAR540* were 0.93 for the C allele and 0.07 for the T allele (Table 5.3). The observed genotype frequency was in Hardy-Weinberg equilibrium.

Table 5.3 the genotype and allele frequencies of selected SNPs of enzymes involved in metabolism of EFV

SNPs	**Genotype Frequency n (%)**			**Allele Frequency %**	
CYP2B6	**GG**	**GT**	**TT**	**G**	**T**
516G>T	226 (29.8)	336 (44.3)	196 (25.9)	52.0	48.0
CYP2B6	**TT**	**TC**	**CC**	**T**	**C**
983T>C	684 (90.5)	71 (9.4)	1 (0.1)	95.2	4.8
CAR540	**CC**	**CT**	**TT**	**C**	**T**
C>T	647 (86.5)	97 (13)	4 (0.5)	93.0	7.0

5.3.3 SNPs impact on plasma EFV concentrations

The impact of each selected SNPs on the mid-dose plasma concentration of EFV is depicted in Figures 5.1 to 5.3. Concentration of EFV was significantly higher in individuals homozygous for the variant allele (TT) at position 516 of the CYP2B6 gene (TT [n=128]: 1800ng/ml vs 1073ng/ml and 929ng/ml for GT [n=226] and GG [n=120] individuals; $p<0.0001$). Similarly, concentration of EFV was significantly higher in individuals homozygous or heterozygous for the variant allele (CC/TC) at position 983 of the CYP2B6 gene ([CC n=1 and TC n=42]: 3235ng/ml vs 1053ng/ml for TT [n=429] individuals; $p<0.0001$). However, the concentration of EFV was not significantly different in individuals homozygous for the variant allele of *CAR540* ([TT] n=1 or heterozygous for the variant allele [CT] n=66 1004.05ng/ml and for normal CC [n=395] individuals 1130ng/ml, p=0.3).

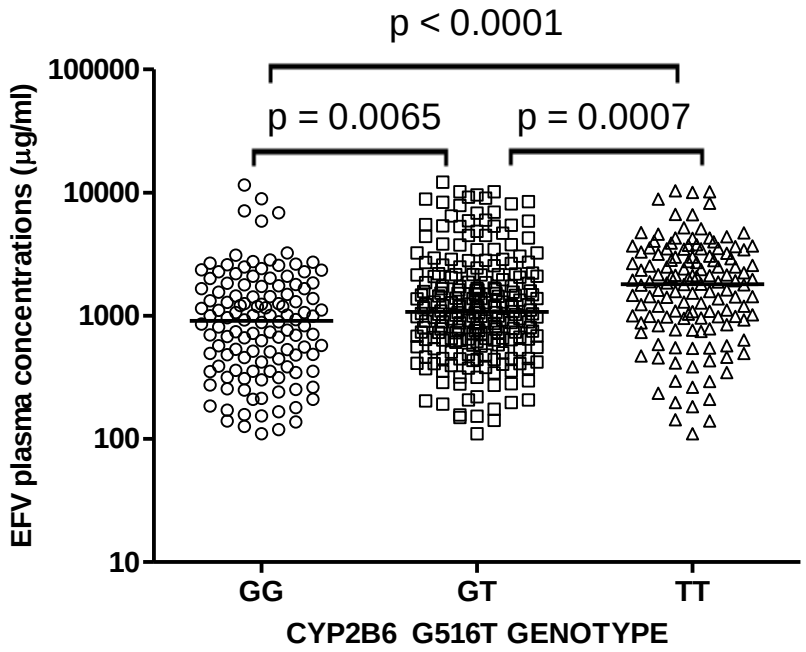


Figure 5.1 the impact of polymorphism of *CYP2B6 516G>T* on the plasma concentrations of EFV. The lines indicate the median values.

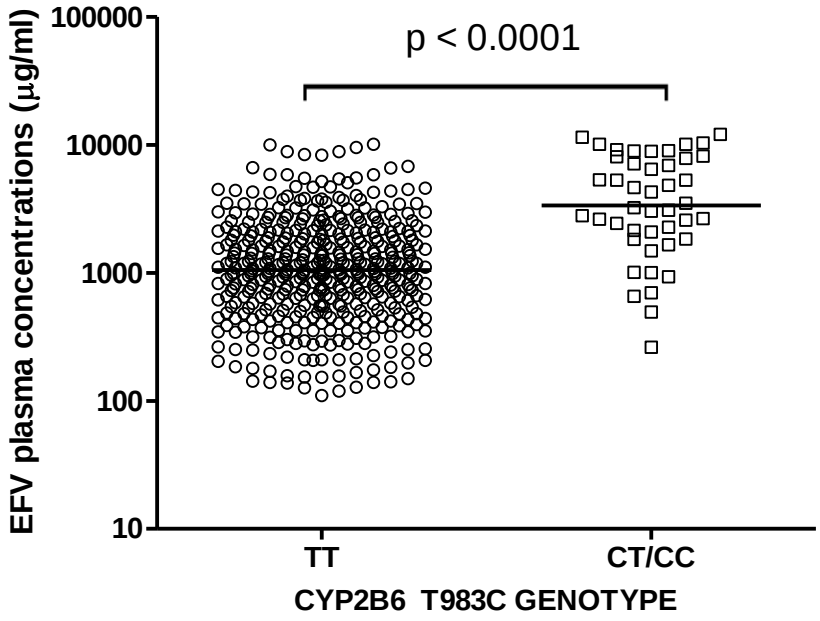


Figure 5.2 the impact of polymorphism of *CYP2B6 983T>C* on the plasma concentrations of EFV. The lines indicate the median values.

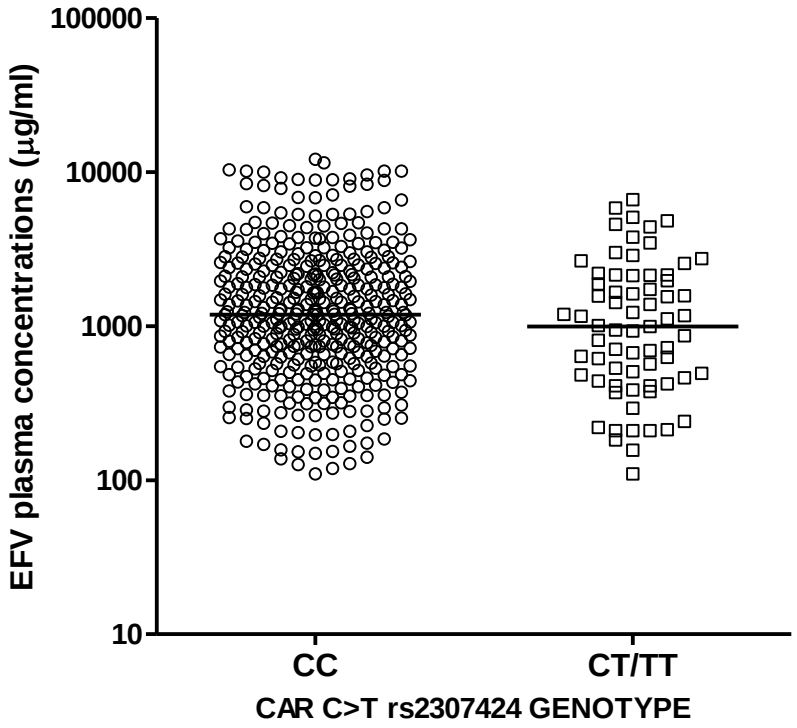


Figure 5.3 the impact of polymorphism of *CAR540 C>T* on the plasma concentrations of EFV. The lines indicate the median values.

5.3.4 CNVs of ABCB5 impact on plasma EFV concentrations

Copy number variation in the ABCB5 gene was identified in 4 patients with a single copy, 197 patients with two copies, 14 patients with three copies, 4 patients with four copies and 1 with six copies. There were no significant associations between ABCB5 copy number and plasma concentrations of EFV (Figure 5.4).

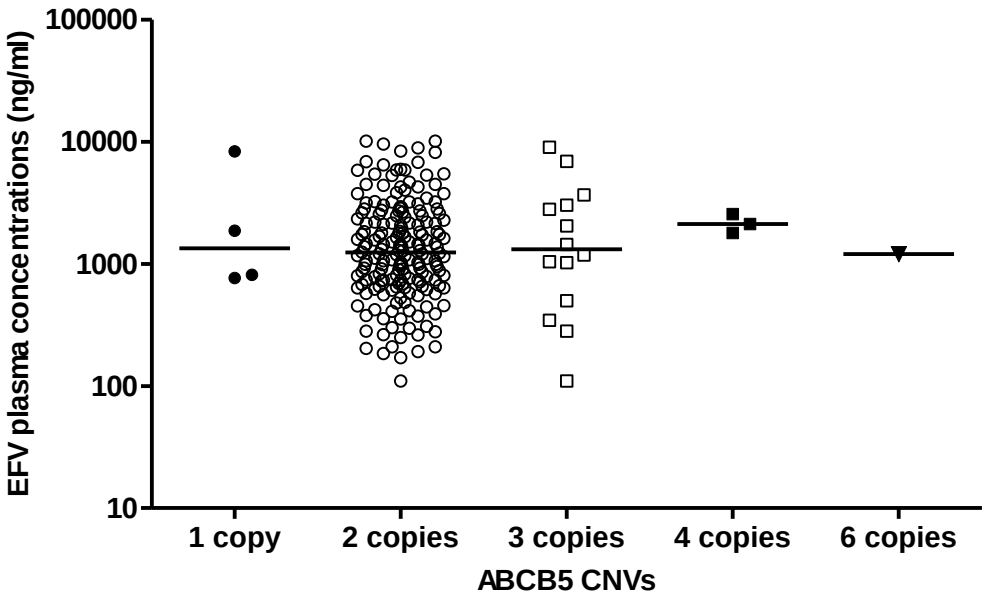


Figure 5.4 the impact of CNVs of ABCB5 on the plasma concentrations of EFV. The lines indicate the median values.

5.3.5 Impact of patient demographics on EFV plasma concentrations

The influence of patient demographics (including gender, age, body weight and height) on EFV plasma concentration was investigated. There was no significant association between gender and EFV plasma concentrations (Figure 5.5). A negative correlation between body weight and EFV plasma concentrations was identified (Figure 5.6). Meanwhile, no other correlations were found between patient demographics and EFV plasma concentration in the Ghanaian cohort (Figure 5.6).

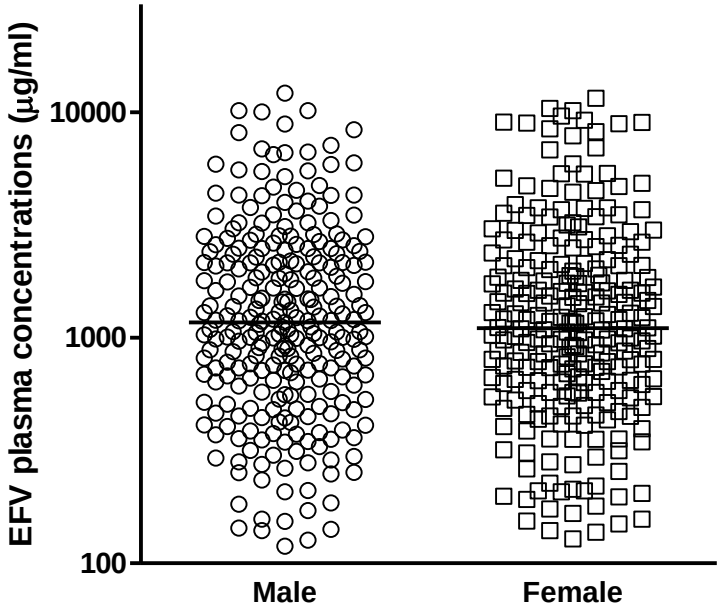


Figure 5.5 Impact of gender on EFV plasma concentration in Ghana cohort. The lines indicate the median values.

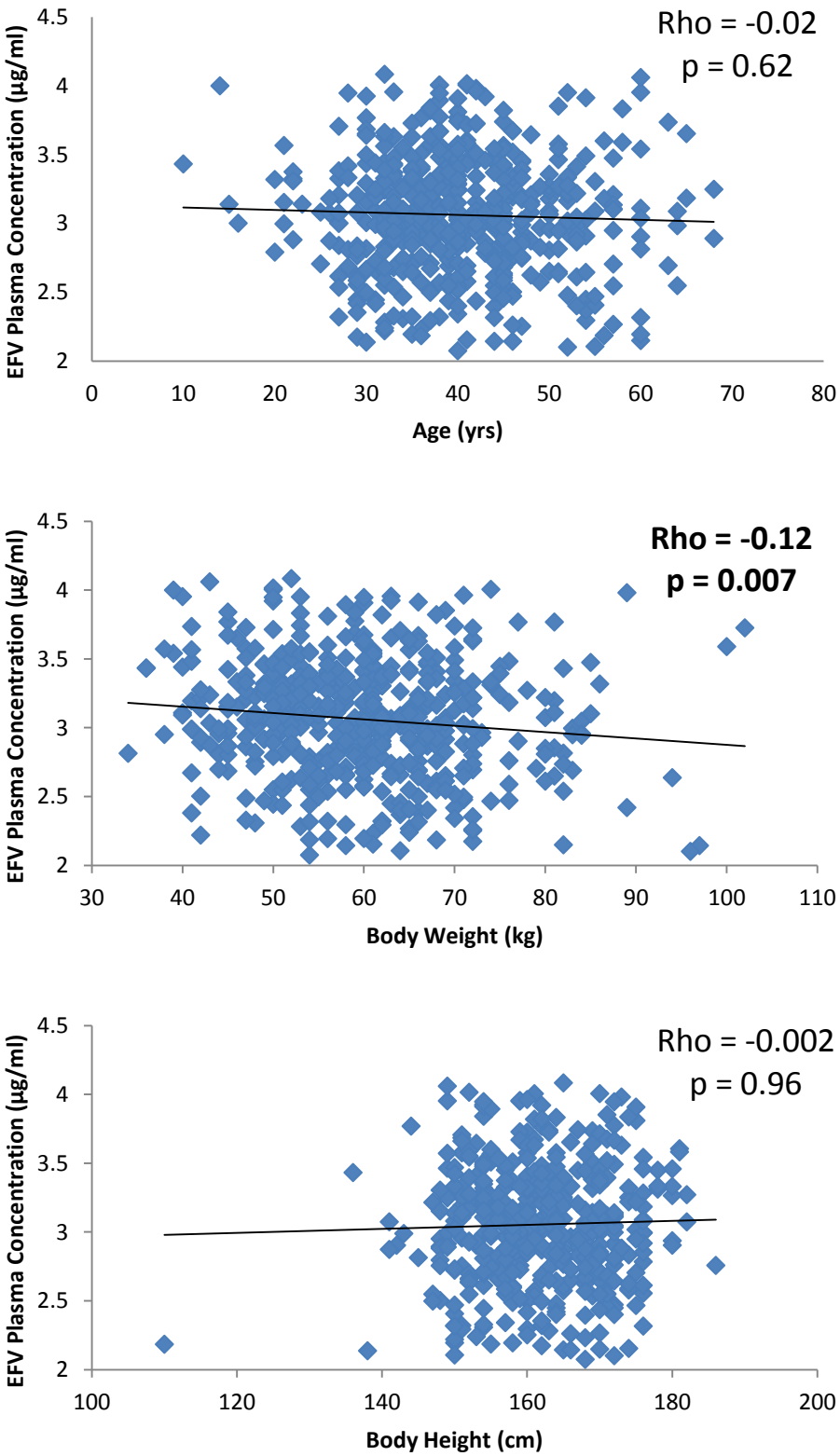


Figure 5.6 Impact of age, body weight, body height with EFV plasma concentration in Ghana cohort

5.3.6 Multivariate analysis

The median plasma concentration of EFV was 1087µg/ml with a range from 110.0µg/ml to 12,146.0µg/ml and a coefficient of variation of 113%. In univariate analysis, body weight in kilograms ($p = 0.008$), polymorphisms in *CYP2B6 G516T* ($p = 2.5 \times 10^{-7}$) and *CYP2B6 T983C* ($p = 4.5 \times 10^{-14}$) were all significantly correlated with log EFV concentration (Table 5.4). In multivariate analysis, body weight, *CYP2B6 G516T* polymorphism and *CYP2B6 T983C* polymorphism were identified as independently associated with EFV exposure as shown in Table 5.4.

Table 5.4 Univariate and multivariate linear regression analyses of independent variables on the log concentration of EFV as dependent variable

Covariate	Univariate P	Multivariate P	Multivariate coefficient (B)
Age (yrs)	0.23	-	-
Gender	0.84	-	-
Weight (kg)	**0.008**	**0.016**	-0.11
Height (cm)	0.45		
CYP2B6 516G>T	**2.5×10^{-7}**	**1.4×10^{-11}**	0.32
CYP2B6 983T>C	**4.5×10^{-14}**	**1.3×10^{-15}**	0.38
CAR540 C>T	0.12	0.07	0.08

5.4 DISCUSSION

CYP2B6 is one of the most polymorphic cytochrome P450 genes in humans with over 100 described polymorphisms and distinct ethnic frequencies (Zanger *et al.*, 2007). Previous studies show that the *CYP2B6 G516T* and *CYP2B6 T983C* polymorphisms are in association with EFV and nevirapine plasma concentration (Calcagno *et al.*, 2011; Haas *et al.*, 2009; Rakhmanina *et al.*, 2010; Rotger *et al.*, 2007; To *et al.*, 2009; Wyen *et al.*, 2008). The *CAR540* polymorphism has previously been shown to influence early treatment discontinuation of EFV in German patients (Wyen *et al.*, 2011). The effect of *CYP2B6 G516T* and *CYP2B6 T983C* was confirmed in this chapter while no significant association emerged for *CAR540* and EFV plasma concentrations. There may be differences in the functionality of this polymorphism between different ethnicities.

A comprehensive analysis of the genotypes frequency has been performed. The frequency of *CYP2B6* (*G516T*) TT was 0.26 in our cohort, similar to previous studies which showed 0.19 in a Ghanaian cohort and 0.23 in a South African cohort (Gounden *et al.*, 2010; Kwara *et al.*, 2009). The T allele frequency of *CYP2B6* (*G516T*) was 0.48 in the current Ghanaian cohort. And the *CYP2B6* (*G516T*) had a frequency of 0.31 in other African populations and 0.16 in Chinese (Brown *et al.*, 2012; Chen *et al.*, 2010). Furthermore, the genotype frequency of *CYP2B6* (*T983C*) TC was 0.09 in the current cohort similarly previous study which identified a

frequency of 0.08 (Kwara *et al.*, 2009).

Copy number variation (CNV) involving deletion or multiplication of DNA segments is a primary source of variation in the human genome (Sebat *et al.*, 2004). In this study, the copy number variation of ABCB5 had no relationship with the plasma concentration of EFV. Although previous studies in our laboratory have shown that EFV is a substrate for ABCB5 (unpublished), the lack of association between CNV and efavirenz PK in this chapter and ABCB5 based upon data in Chapter 6 indicated that efavirenz is unlikely to be a transported substrate of ABCB5.

Meanwhile, no correlation has been found between ABCB5 genetics and EFV plasma concentrations in the literature. However, several studies have investigated whether CCL3L1 CNV influences the risk of HIV-1 infection, but there are no studies that have shown CNVs to be important for HIV drugs (Liu *et al.*, 2010). Recent studies have suggested an increasing role for CNVs in many diseases (Fanciulli *et al.*, 2010; Levy *et al.*, 2012) because a number of genes involved in drug metabolism exhibit CNVs (Gaedigk *et al.*, 2012). For example, CYP2D6 CNVs can result in reduced or increased metabolism of many clinically used drugs (Sheng *et al.*, 2007). The investigation of CYP2D6 allelic distributions and CNVs in ethnic populations can be an important determinant for future dose optimization (Gaedigk *et al.*, 2011; Kim *et al.*, 2010; Kim *et al.*, 2012; Sheng *et al.*, 2007).

Age, gender, body weight and height are also important factors which may influence drug pharmacokinetics. In this study, a negative correlation was found between the body weight and the EFV plasma concentration in univariate analysis. Previous studies showed a significant and inverse correlation between efavirenz concentrations and body weight in Brazilian and Thai cohort (Manosuthi *et al.*, 2009; Poeta *et al.*, 2011). Obesity was also reported that it represented a risk factor for antiretroviral therapy underdosing (de Roche *et al.*, 2012)

In conclusion, the polymorphisms of body weight, *CYP2B6 G516T* and *CYP2B6 T983C* affect EFV plasma concentrations in Ghanaian patients. However, *CAR540* did not exert a significant impact on EFV exposure in this cohort of Ghanaian HIV-1 infected patients on EFV-based therapy. The lack of association between CNV and efavirenz pharmacokinetics support ABCB5 does not substrate for efavirenz.

CHAPTER 6

Determination of Nano antiretroviral drugs transcellular permeability in Caco-2, MDCKII and MDCKII-ABCB5 cell lines

Contents

6.1 INTRODUCTION

As in other diseases, development of new antiretroviral drug candidates is severely hampered by poor solubility. Insolubility is limited not only to new molecules but also prevents many existing therapies from optimally performing. Nanotechnology represents a major opportunity to enhance the performance of existing medicines. New non-attrition techniques have been developed in Liverpool to produce Nanomedicines that overcome some limitations associated with conventional formulations. Nanoparticle preparation is currently subject to patent fillings and cannot be disclosed. However, they were produced using a previously published synthesis (Zhang *et al.*, 2008). Application of these techniques to current antiretroviral drugs offers the potential to generate next generation therapies with enhanced clinical and patient benefits.

The majority of antiretroviral drugs are orally administered and their absorption from the gastrointestinal tract is pivotal for their success in therapy. The rate and extent of intestinal absorption of drug products is dependent upon physicochemical (e.g. solubility, size, charge and surface area), physiological (e.g., stomach emptying, intestinal motility and membrane permeability), formulation (e.g., dosage form, dissolution, excipients and stability) and protein activity (e.g., metabolism, efflux and uptake) (Volpe, 2011). Membrane permeability is one of the most important factors in intestinal absorption and can be investigated using specific cell lines such as Caco-2

and Madin–Darby canine kidney (MDCK) cells.

The Caco-2 cell line, isolated from a human colon adenocarcinoma, differentiates into enterocytes and forms polarized monolayers of columnar epithelial cells with tight junctions and a brush border and are actively used as a model by industry (Elsby *et al.*, 2008). Caco-2 cell monolayers have high transepithelial electrical resistance (TEER) values and the advantage of containing both passive and active (uptake, efflux) permeability mechanisms (Table 6.1). The MDCK cells, isolated from canine distal renal tissue, also differentiate into columnar epithelium and form tight junctions. The MDCK cells have lower TEER and shorter culture times than Caco-2 cells (Table 6.1). The Caco-2 and MDCK cell lines have a relationship to human intestinal absorption and drug permeability as they can be used to model passive diffusion (paracellular, transcellular), carrier-mediated uptake, and carrier-mediated efflux and so on. Therefore, they are rapid tools to screen the drug permeability and transport at the cellular level. However, there are some limitations to using them because some substances have very low aqueous solubility, toxic drugs adversely affect results of permeability measures, physiological factors are difficult to predict and proteins and transporters can have different expression in primary cells compared to these cell lines (Zheng *et al.*, 2011).

Caco-2 cells have been shown to express both efflux (ABCB1, ABCG2 and ABCC subfamily of transporters) and influx (multiple proteins of OATP subfamily of transporters, PepT1, OCT1-3) transporters (Majer *et al.*, 2012; Mease *et al.*, 2012; Prime-Chapman *et al.*, 2004; Seithel *et al.*, 2006). MDCK cells express ABCB1(Mease *et al.*, 2012).

Very little is known about transporters capable of influx and efflux of efavirenz (EFV) in intestine or liver. Recent work in Liverpool has indicated that EFV may be a substrate for ABCB5 (unpublished observation). ABCB5 is an ATP-binding cassette (ABC) efflux transporter that is of the same sub-family as ABCB1 (Frank *et al.*, 2005). like ABCB1, ABCB5 has been shown to be expressed in intestine (Taipalensuu *et al.*, 2001) and may therefore influence absorption of EFV. However, it should be noticed that in chapter 5, no association of ABCB5 CNVs with EFV plasma concentration was observed in patients from Ghana.

In this chapter, the Caco-2 cell line was used to evaluate the permeability of ten nanodispersions of lopinavir (LPV) (designated NanoLPV1-NanoLPV10) compared with an aqueous solution of LPV. Meanwhile, the transcellular permeability of six nanodispersions of efavirenz (designated NanoEFV1 to NanoEFV6) was compared with an aqueous solution of EFV in MDCKII as well as the MDCKII-ABCB5 (ABCB5-overexpressing) cell line.

Table 6.1 Cell line characteristics

	Caco-2	**MDCK**
Cell source	Human colon adenocarcinoma	Canine kidney
Cell morphology	Intestinal epithelium	Distal tubule epithelium
Cell culture time	14–28 days	3–7 days
TEER value	600	200

6.2 METHODS

6.2.1 Materials

The Caco-2 cell line was purchased from European collection of cell cultures (ECACC No.286010202). The MDCKII cells were a kind gift from Prof. P. Borst (Amsterdam Netherlands). And the cells were counted using a NucleoCounter (ChemoMetec, Denmark) cell counter. MDCKII-ABCB5 was developed in-house as part of a separate project. A Millicell Electrical Resistance System (Fisher Scientific, Leicestershire, UK) was used for measuring Monolayer TEER. LPV was provided by Abbott Laboratories (Chicago, USA), EFV by DuPont Bristol Myers Squibb (New Brunswick, NJ, USA). Radiolabelled LPV (^{3}H LPV) and EFV (^{14}C EFV) were purchased from Moravek Biochemicals, Inc. (Brea California, USA). Dulbecco modified Eagle medium (DMEM), foetal bovine serum (FBS), Hanks buffered salt solution (HBSS) and Trypsin-EDTA solution were purchased from Sigma Aldrich (Poole, UK). Ultima Gold liquid scintillation cocktail was obtained from Packard (Groningen, Netherlands).

6.2.2 Cell culture

Caco-2 cells were cultured in DMEM supplemented with FBS (15%v/v) and MDCKII and MDCKII-ABCB5 cells were grown in DMEM supplemented with L-glutamine, penicillin/streptomycin (1%v/v) and FBS (10%v/v). The cells were grown and routinely seeded in tissue culture treated flasks in a humidified chamber (37 ℃, 10% CO_2 incubator) and harvested by regular trypsinisation. The medium was changed every 2 to 3 days until the confluence of the cell monolayer was achieved. Trypsinisation involved decanting the media, followed by washing twice with 20 ml HBSS and the detachment of the monolayer by addition of 10ml Trypsin-EDTA. The cells were then incubated for 10 minutes. The resulting suspension was centrifuged (2000 g × 5 min, 4 ℃), the supernatant removed and the resulting pellet re-suspended in 10 ml of fresh DMEM, counted using a cell counter and ~ 7 million cells transferred to each new flask and made to 35 ml with DMEM.

6.2.3 Storage of Cells

The cells were trypsinised as described earlier after attaining confluence (section 6.2.2). The pellets were then re-suspended in DMEM, counted using a cell counter and centrifuged (2000 g × 5 min, 4 ℃). The cells were then re-suspended in warm FBS (FBS + 10%v/v DMSO), mixed thoroughly and made up to a concentration of 5 $\times 10^6$ cells per ml. 1 ml of cell suspension was then transferred to pre-labelled 1.5 ml cryovials and frozen at -80 ℃ for use as and when required. The viable semi-frozen cells were thawed by placing the cryovials rapidly in a waterbath (37 ℃) or by simply holding the vials in the hands for a few minutes and re-suspending the pellets in 9 ml DMEM followed by culturing as described in section 6.2.2.

6.2.4 Cell seeding

The cells were trypsinised as described in section 6.2.2, and after centrifugation the pellet was re-suspended in fresh DMEM, and the cells counted using a cell counter. A volume of DMEM was added to give a cell count of 1.4×10^5 cells per ml, and cells were seeded on transwell culture plates at density of ~35,000 cells per well for the Caco-2 cell line and ~100,000 cells per well for the MDCKII and MDCK-ABCB5 cell lines. The plates were then incubated at 37℃ and 10% CO_2 in a humidified chamber and the media was changed every 2-3 days, by aspirating using a suction pump and replacing with an equal volume of DMEM. Caco-2 cell monolayers were used for transport studies 15–21 days post seeding over passage numbers 27–36, while MDCKII and MDCK-ABCB5 cell lines were used for transport studies 7–12 days post seeding over passage numbers 10–16. The TEER across the cell monolayer was monitored using a Millicell Electrical Resistance System to assess cell monolayer integrity and the monolayer was considered appropriate for experiments when the TEER values were typically above 600 cm^2 for the Caco-2 cell line and above 200 cm^2 for MDCKII and MDCKII-ABCB5 cell lines.

6.2.5 Permeability assays

Prior to transport studies, the medium was removed from all apical and basolateral compartments of the transwells and washed with HBSS. Cell monolayers were pre-incubated in transport buffer (HBSS containing 25mM HEPES and 0.1% (w/v) bovine serum albumin, pH 7.4) for 10–15 min at 37℃ prior to the addition of donor and receiver solutions. Following this pre-incubation period, HBSS containing 10μM of the desired drug was added to the donor compartments of transwells situated on either the apical (total volume = 250 ml) or basolateral (total volume = 600 ml) side of the monolayer. Transport buffer was added to the corresponding receiver compartments. Permeability in each direction was done in quadruplicate. The transport of the compound across the monolayer is monitored over a 60 min time period in incubator (37℃, 10% CO_2 incubator), and 150μl of samples from the apical (basolateral to apical) or basolateral (apical to basolateral) compartments were taken and quantified by using a liquid scintillation counter.

6.2.6 Statistical analysis

The apparent permeability coefficient (P_{app}) is determined from the amount of compound transported per time. The values are expressed as cm/second and are calculated according to the following equation:

$P_{app} = ((dQ/dt) \times V) / (A \times C_0)$

Where dQ is the concentration variations, dt is the time variations, V is the sample volume (0.6 ml for apical to basolateral, 0.25 ml for basolateral to apical), A is the exposed surface area (0.3 cm^2) and C_0 is concentration at the start (10μM).

The results were presented as mean ± standard deviation (SD) of four experiments with 95% confidence intervals for differences between the means where appropriate. The means of P_{app} were then used to calculate efflux ratio (ER = Papp basolateral-to-apical/Papp apical-to-basolateral)

6.3 RESULTS

6.3.1 Impact of nanoformulation on the permeability of LPV in Caco-2 cells

The bi-directional P_{app} of ten nanodispersions of LPV (NanoLPV1 to NanoLPV10) and an aqueous solution of LPV in Caco-2 cells were shown in figure 6.1. Only two nanodispersions (NanoLPV6 and 9) had a higher permeability than aqueous LPV not only in basolateral to apical direction but also in the apical to basolateral direction. All nanodispersions of LPV (except NanoLPV4) had a higher permeability than the aqueous LPV in the apical to basolateral direction. However, other nanodispersions of LPV (NanoLPV1, 2, 3, 4, 5, 7, 8 and 10) exhibited a lower permeability than aqueous LPV in basolateral to apical direction. The efflux ratio of aqueous LPV was 1.1, while efflux ratios of LPV nanodispersions were ranged from 0.41 to 0.95.

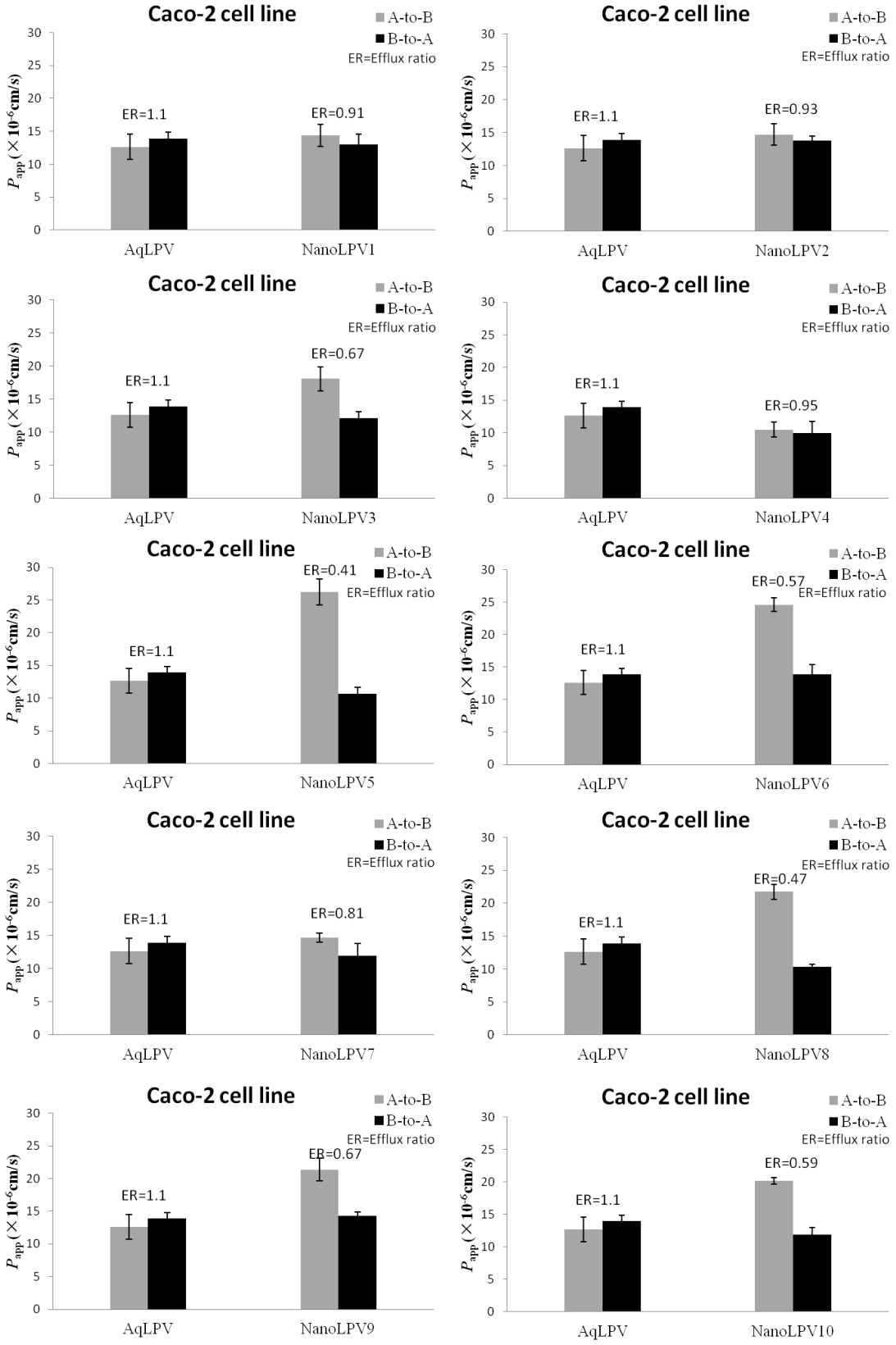


Figure 6.1 comparing the permeability of nanodispersions and aqueous LPV in the Caco-2 cell line.

6.3.2 Impact of nanoformulation on the permeability of EFV in MDCKII and MDCKII-ABCB5 cells

The bi-directional P_{app} of six nanodispersions of EFV (NanoEFV1 to NanoEFV6) and an aqueous solution of EFV in MDCKII and MDCKII-ABCB5 cell lines were shown in figure 6.2 and 6.3. All nanodispersions had a lower permeability than the aqueous EFV in both apical to basolateral and basolateral to apical directions by using MDCKII cells. In MDCKII-ABCB5 cell lines, the nanotechnology had a negative effect on EFV permeability in both apical to basolateral and basolateral to apical directions in the MDCKII-ABCB5 cell lines (Figure 6.3). However, NanoEFV4 had a higher permeability than the aqueous EFV in apical to basolateral direction (Figure 6.3). The efflux ratio of aqueous LPV in MDCKII and MDCKII-ABCB5 cell lines was 0.18 and 0.15, respectively. The efflux ratios of LPV nanodispersions were ranged from 0.10 to 0.23 in MDCKII cell lines and 0.05 to 0.21 in MDCKII-ABCB5 cell lines.

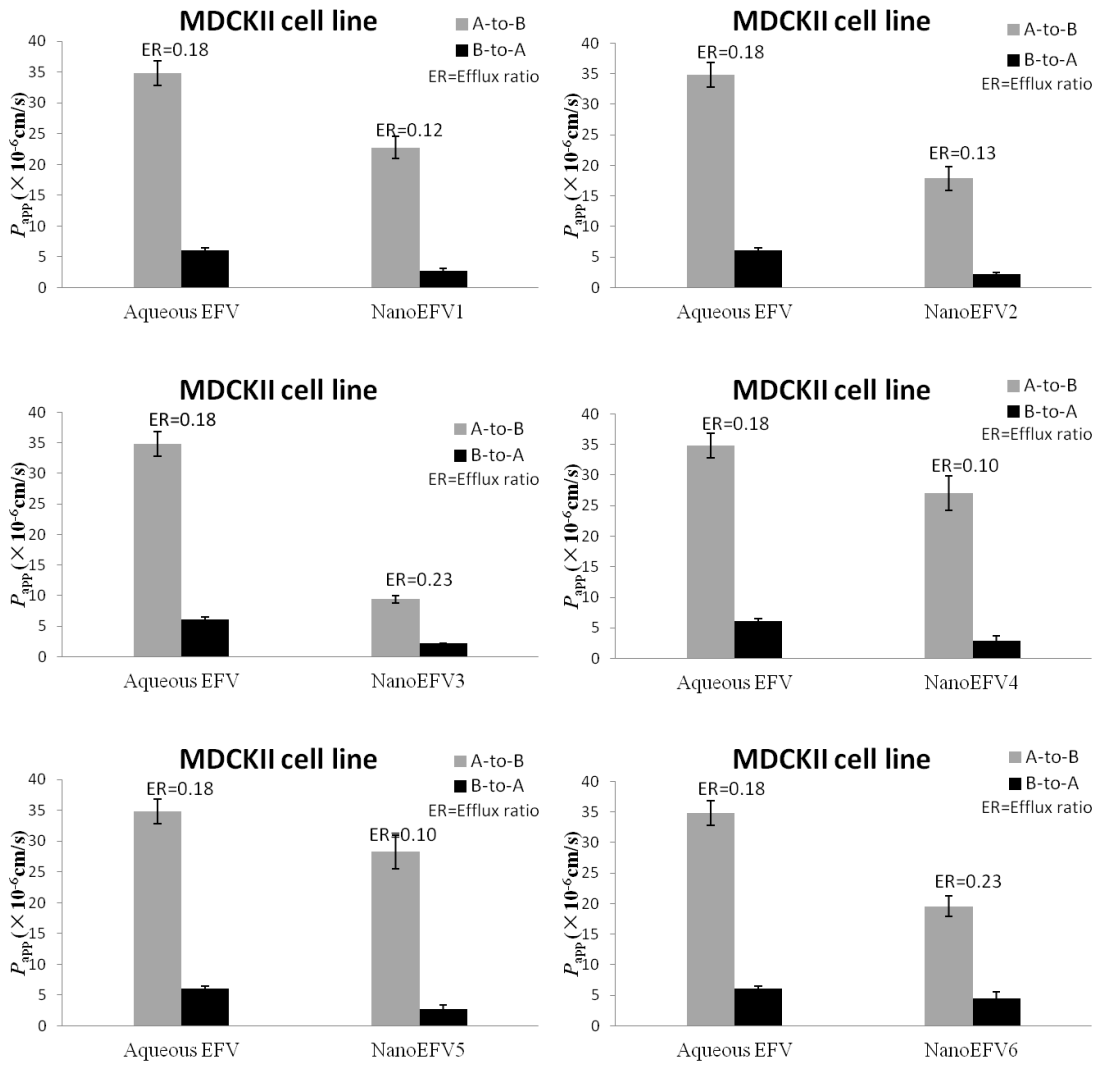


Figure 6.2 comparing the transcellular permeability of nanodispersions and aqueous EFV in the MDCKII cell line.

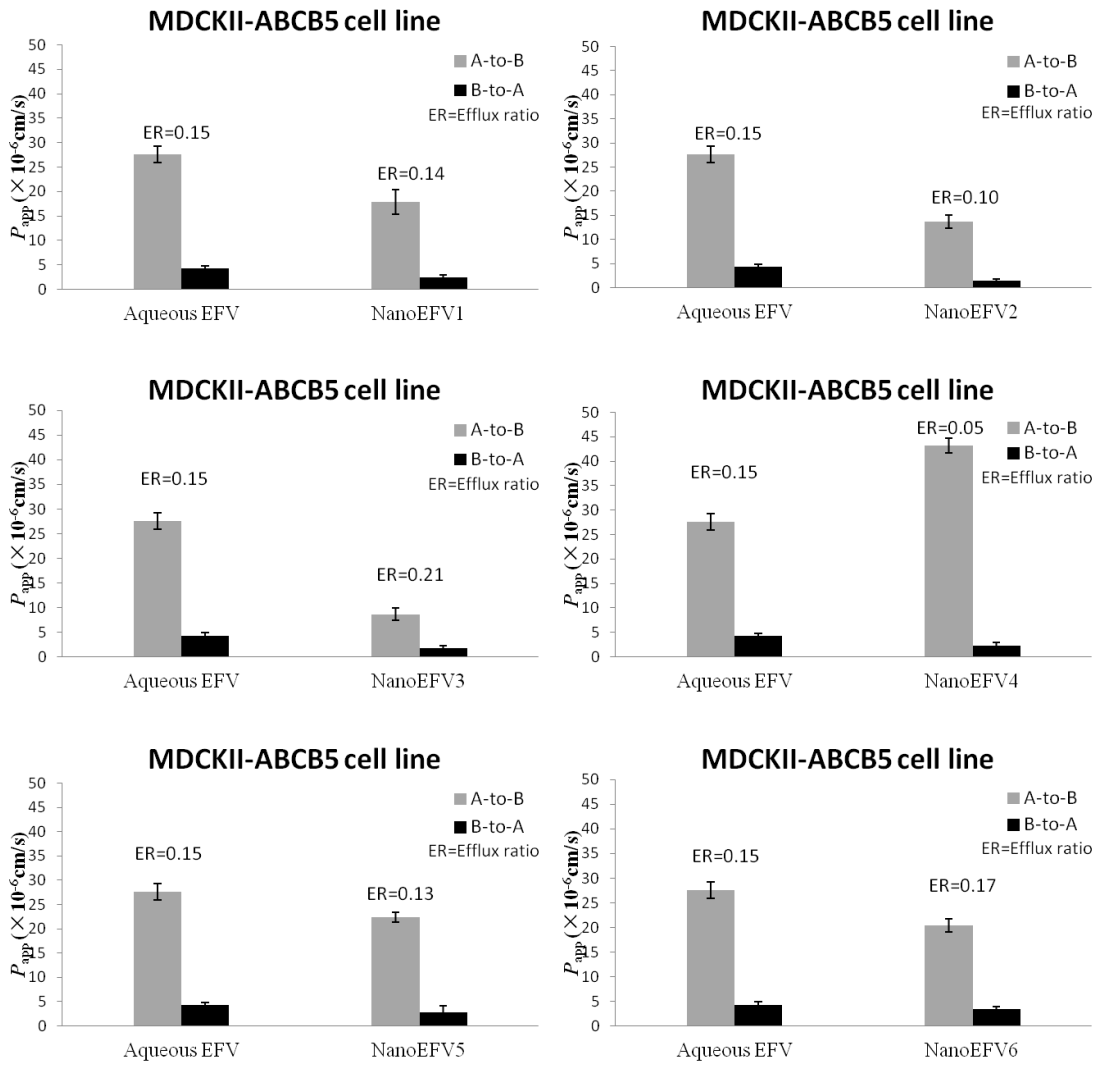


Figure 6.3 comparing the permeability of nanodispersions and aqueous EFV in the MDCKII-ABCB5 cell line.

6.3.3 Impact of ABCB5 on the permeability of nanodispersions and aqueous solution of EFV

The bi-directional P_{app} of nanodispersions of EFV and an aqueous solution of EFV in MDCKII and MDCKII-ABCB5 cell lines were shown in figure 6.4. The bi-directional permeability of EFV nanodispersions (NanoEFV1-3) and aqueous EFV were higher in both MDCKII cell lines than the permeability in MDCKII-ABCB5 cell lines. Two nanodispersions of EFV (NanoEFV4 and 6) had a lower permeability in MDCKII cell lines from apical to basolateral direction but a higher permeability from basolateral to apical direction. Conversely, the permeability of NanoEFV5 was higher in MDCKII cell lines from apical to basolateral direction but a lower from basolateral to apical direction. ABCB5 increased the permeability of Two nanodispersions of EFV (NanoEFV4 and 6) from apical to basolateral direction were observed. Conversely, the permeability of NanoEFV5 was increased by the ABCB5 from basolateral to apical direction. The efflux ratios of EFV nanodispersions and aqueous EFV were ranged from 0.10 to 0.23 in MDCKII cell lines and 0.05 to 0.21 in MDCKII-ABCB5 cell lines.

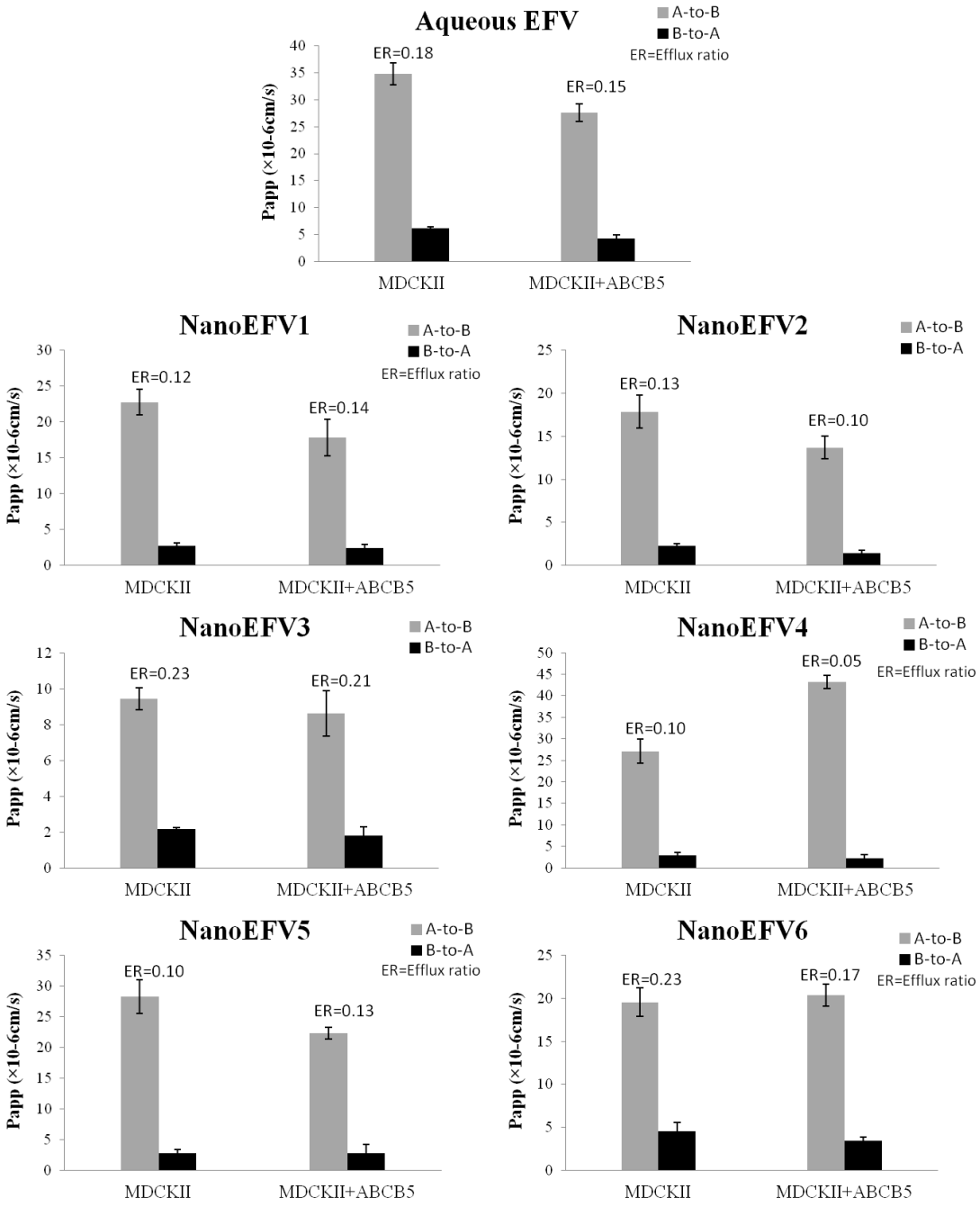


Figure 6.4 comparing the permeability of nanodispersions and aqueous EFV in MDCK and MDCKII-ABCB5 cell line.

6.4 DISCUSSION

The aim of this chapter was to investigate the impact of Nanomedicine on the permeability of antiretroviral drugs in cell lines. LPV permeability in the Caco-2 cell line was increased by nanoformulation into nanodispersion. 90% of the nanodispersions had a higher permeability than aqueous drug in apical to basolateral direction. 10% of LPV were increased permeability by nanoformulation in both apical to basolateral and basolateral to apical directions. Meanwhile, the bi-directional permeability of EFV was decreased by nanoformulation in both MDCKII and MDCKII-ABCB5 cell lines.

In a previous study, some other nanotechnologies have been used to enhance drug performance. For example, lipid nanocarriers improved paclitaxel transport through Caco-2 cell monolayers by using vesicle-mediated transcytosis (Roger *et al.*, 2009). Permeability experiments of Sadighi *et al* revealed that furosemide/HP-betaCD and ranitidine HCl/HP-betaCD nanoformulations greatly induced the opening of tight junctions and enhanced drug transition through Caco-2 monolayers (Sadighi *et al.*, 2012).

Furthermore, we also compared efficiency of EFV permeability in MDCKII and MDCKII-ABCB5 cell lines and ABCB5 decreased the bi-directional permeability of EFV were observed. Although previous studies in our laboratory have shown that

EFV is a substrate for ABCB5 (unpublished). The data presented in this chapter indicate that ABCB5 is not able to transport EFV neither nanodispersions nor aqueous solution. In previous study, the MDCKII-ABCB1 and MDCKII-ABCC2 cells have significantly increased LPV efflux ratio relative to the parental cells due to the apically directed transport by ABCB1 and ABCC2, respectively (Agarwal *et al.*, 2007). However, the study of Janneh *et al* showed that ABCB1 expression had no effect on the accumulation of EFV and nevirapine in MDCKII and MDCKII-ABCB1, while ABCC1/2 expression and OATP-like transporters (possibly OATP3A1) may have greater influence on the accumulation of EFV in MDCKII and MDCKII-ABCB1 (Janneh *et al.*, 2009).

In conclusion, nanoformulation improved LPV permeability across the Caco-2 cell line but decreased the permeability of EFV across MDCKII and MDCKII-ABCB5 cell lines. ABCB5 is not able to transport EFV neither nanodispersions nor aqueous solution. The ability to change permeability of drugs based on the formulation of solid drug nanoparticles is worthy of further study in order to improve drug delivery and drug distribution.

CHAPTER 7

Concluding Discussion

Current antiretroviral therapy consists of a combination of at least three drugs and has dramatically improved the prognosis of patients infected with HIV. However, viral replication during therapy can lead to the selection of drug resistant viruses (Cressey *et al.*, 2007). Resistance to antiretroviral therapy has been documented for every class of antiretroviral drug including NRTIs, NNRTIs, PIs and inhibitors of viral fusion, entry and integration (Boyd *et al.*, 2010). Therefore, currently available therapy aims to suppress HIV replication for as long as possible thus delaying the progressive destruction of the immune system and postponing the occurrence of opportunistic infections and the onset of AIDS. The failure of antiretroviral therapy may be due to pharmacological factors such as first pass metabolism and inadequate penetration into HIV replication-competent cells. This thesis investigated the pharmacological factors such as drug transporters (efflux and influx) and metabolism enzymes.

Drug transporters and metabolism enzymes played complementary roles in drug absorption, distribution, metabolism and excretion by biotransformation and counter-transport, particularly in the intestine (Benet, 2009), while nuclear receptors as transcription factors regulate the expression of drug transporters and metabolism enzymes. In chapter 2, a positive correlation between nuclear receptors expression and the expression of ABC transporters and OATP transporters in intestine were observed. According to the Les Benet's study, when nuclear receptors increased the expression of ABC transporters these efflux transporters increased presentation of drug to CYP3A4 and more metabolism as a result of cycles of efflux (Benet, 2009).

Nuclear receptors also improved the expression of influx OATP transporters to increased presentation of drug to CYP3A4. However, a significant negative correlation was found between the gene expression of nuclear receptors and cytochrome P450 enzymes in intestine in chapter 3. In previous study, positive correlation between the gene expression of nuclear receptors and cytochrome P450 enzymes in liver has been reported (Chang *et al.*, 2003; Lamba *et al.*, 2010; Vyhlidal *et al.*, 2006). The reasons for this difference between liver and intestine may involve differences between expressions of other transcription factors such as Vitamin D between these tissues. In literature, PXR dominantly controls CYP3A4 inducibility in the liver, whereas Vitamin D receptor transactivates CYP3A4 in the intestine by secondary bile acids (Pavek *et al.*, 2010).

There have been a number of recent studies that investigate polymorphisms of genes that could potentially impact on gene expression of drug transporters and metabolism enzymes. The *PXR63396* polymorphism has been reported to be associated with PXR and CYP3A4 gene expression (Schipani *et al.*, 2010). PXR promoter and intron 1 SNPs are also associated with PXR target gene expression (CYP3A4) in donor livers and cultured hepatocytes (Lamba *et al.*, 2008). Another PXR polymorphism (*PXR A566C*) was significantly associated with CYP3A4 RNA expression in colon tumor (King *et al.*, 2007). *CYP2B6 C1459T* significantly reduced CYP2B6 expression in liver (Lang *et al.*, 2001), while *CYP3A4 A392G* has been shown to be in an association with CYP3A4 gene expression (Svard *et al.*, 2010). *CYP2B6 C1459T* was

also associated with the lowest level of CYP2B6 activity in the livers of females (Lamba *et al.*, 2003). In chapter 2, a statistically significant association emerged between *PXR63396* polymorphism and the expression of ABCB1 and ABCC2 in intestine and *PXR44477* polymorphism was associated with ABCC10 expression in intestine. However, no impact of *PXR44477*, *PXR69789* and *PXR63396* and *CAR C>T* (rs2307424) polymorphisms on the gene expression of OATP transporters and cytochrome P450 enzymes in intestine was found. The polymorphism of *CYP2B6 (G516T* and *C1459T)* and *CYP3A4 A392G* also had no influence with the gene expression of cytochrome P450 enzymes. A clear understanding of the relationship between polymorphisms and expression of these proteins is important to rationalise differences observed in pharmacokinetics of relevant substrates.

High inter-individual variability in drug concentrations has been demonstrated in patients receiving the same dose of antiretroviral drugs (Back *et al.*, 2006). For example, EFV interpatient concentration variation is 118% (Marzolini *et al.*, 2001). There are many factors that may affect plasma concentrations such as age, gender, body weight, ethnicity, genetic factors and so on. Plasma concentrations of the NNRTIs have been shown to correlate with virological response (Fabbiani *et al.*, 2009). Therefore, factors that influence the variability in plasma concentrations may cause patients to experience therapeutic failure or toxicity (Justesen, 2006). Chapter 4 and 5 investigated the factors that influence EFV plasma concentrations in UK and Ghanaian cohorts.

EFV plasma concentrations are often used to optimise therapy by ensuring minimum effective plasma concentrations are achieved (Sun *et al.*, 2010; Yimer *et al.*, 2011). Age, gender, body weight and height are important factors which may influence drug pharmacokinetics. In a Ghanaian cohort, a negative correlation was found between the body weight and the EFV plasma concentration in univariate analysis. It is support by previous studies in Brazilian and Thai cohort (Manosuthi *et al.*, 2009; Poeta *et al.*, 2011). Obesity was also reported that it represented a risk factor for antiretroviral therapy underdosing (de Roche *et al.*, 2012). The genetic factors including polymorphisms of *CYP2B6 G516T* and *CYP2B6 T983C* influenced EFV plasma concentrations while the polymorphism of *CAR C>T* (rs2307424) in the Ghanaian cohort. Similarly, *CYP2B6 G516T* and *CYP2B6 T983C* polymorphisms were shown to be associated with plasma concentrations of EFV in previous studies (Rakhmanina *et al.*, 2010; To *et al.*, 2009; Wyen *et al.*, 2008). In the UK cohort, EFV plasma concentrations were also found to be associated with viral load in plasma.

One of the reasons for antiretroviral therapy failure is the inability of drugs to reach viral reservoir sites such as macrophages (Vyas *et al.*, 2006). This may result in relapses and increase likelihood of resistance. Nanodispersion technology is a new promising technology that may enable and improve the targeted delivery of antiretroviral drugs (Emerich *et al.*, 2006). LPV transcellular permeability in the Caco-2 cell line was improved by formulation into nanodispersions. However, for EFV the transcellular permeability was decreased for all nanodispersions in MDCKII

and MDCKII-ABCB5 cell lines. Comparing efficiency of EFV nanodispersions permeability in MDCKII and MDCKII-ABCB5 cell lines indicated that ABCB5 is not able to transport EFV when incubated as nanodispersion or dissolved molecule. It is support by the copy number variation of ABCB5 had no relationship with EFV plasma concentrations in Chapter 5. The MDCKII-ABCB1 and MDCKII-ABCC2 cells significantly increased LPV efflux ratio relative to the parental cells due to the apically directed transport by ABCB1 and ABCC2 respectively (Agarwal *et al.*, 2007). ABCC1/2 expression and the OATP3A1 transporter may have greater influence on the accumulation of EFV in MDCKII and MDCKII-ABCB1 (Janneh *et al.*, 2009)

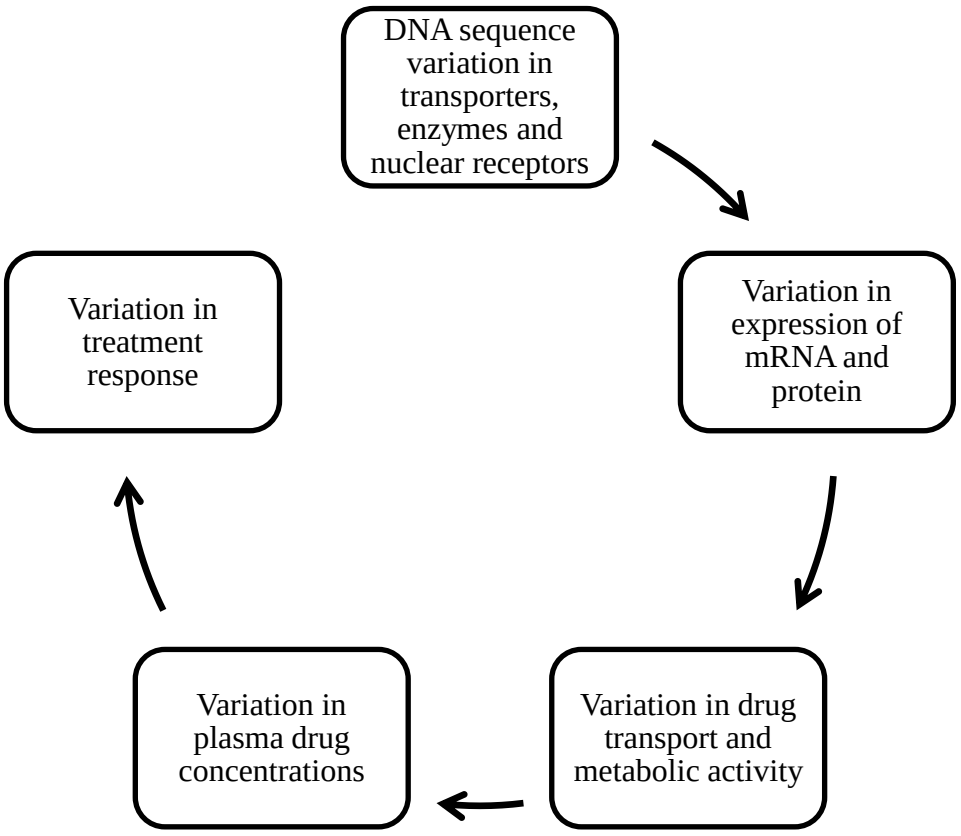


Figure 7.1 Variations of pharmacological factors that affect treatment response.

In summary, this thesis examines pharmacological factors involved in antiretroviral therapy. Figure 7.1 summarises how these factors impact on the variation in treatment response. The variability of DNA sequence in drug transporters, metabolism enzymes and nuclear receptors associates with the expression of mRNA and protein. In turn, this may impact upon the drug transport and metabolic activity. It also presents data that shows the factors that influence drug concentrations, which are important for viral suppression. Finally, nonodispersion technology is worthy of further study in order to improve drug delivery and distribution.

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